P53 pathway alterations in breast cancer for biomarker discovery

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1. GENERAL INTRODUCTION

1.1 Breast cancer

The high incidence of cancer in both developed and developing countries is a remarkable leading cause of global death. Breast cancer prevalence is estimated at 23% and 14% mortality among total cancers accounting for female cancers worldwide[1]. The high incidence of breast cancer is mainly associated with hormonal factors and adaption of western life style including physical inactivity, obesity and late age at first birth[2, 3]. The mortality rate of breast cancer has markedly decreased with the advantage of mammography and the utilization of advanced treatment strategies [4]; however, more detection methods such as novel biomarkers and new therapeutic strategies are needed to be developed.

1.1.1 Breast cancer subtypes

Classification of breast cancer into subtypes is primarily used to aid in treatment and prognosis. Breast cancer is generally categorized into two main histopathological classes, in situ carcinoma and invasive/infiltrating carcinoma. In situ carcinoma is further classified into two subtypes as Ductal carcinoma in situ (DCIS), the most common form of breast cancer, and lobular carcinoma in situ (LCIS), which encompasses a heterogeneous group of tumors. Invasive/infiltrating carcinoma is divided to seven subtypes as illustrated in Table 1[5].

In addition to the conventional histologic parameters, classification based on molecular components has been described with greater prognostic values for managing target therapy in breast cancer. Molecular subtypes of breast cancer are categorized as basal-like, ErbB2+, normal breast like, luminal subtype A and luminal subtype B, and claudin low. These subtypes could serve as predictive indicators for overall survival and disease-free survival[6-10].

Table.1 Histological classification of breast cancer subtypes.

Breast cancer	Sub-types
in situ carcinoma	Ductal
	lobular
invasive/infiltrating carcinoma	Tubular
	Ductal lobular
	Invasive Lobular
	Infiltrating Ductal
	Mucinous (colloid)
	Medullary

1.1.2 Lymph node metastasis

Invasion and metastasis are important hallmarks of malignant tumors associated with complex genetics and epigenetic alterations that allow tumors to disseminate throughout lymphatic or blood vessels, giving rise to the colonization and growth of metastatic cells in distant organs[11-13]. Breast cancer, a heterogeneous disease, presents various pathological signs such as axillary lymph node metastasis (Figure 1.), which is associated with a high risk of recurrence and considered as an important prognosis factor in early stages of the disease[14, 15]. Several studies have tried to identify primary tumor signatures correlated to metastasis. Using microarray analysis, sets of prognostic genes (the Amsterdam signature, 70 genes, and the Rotterdam signature, 76 genes) were established that predicted the development of distant metastasis within 5 years in primary breast cancer patients[16-18].

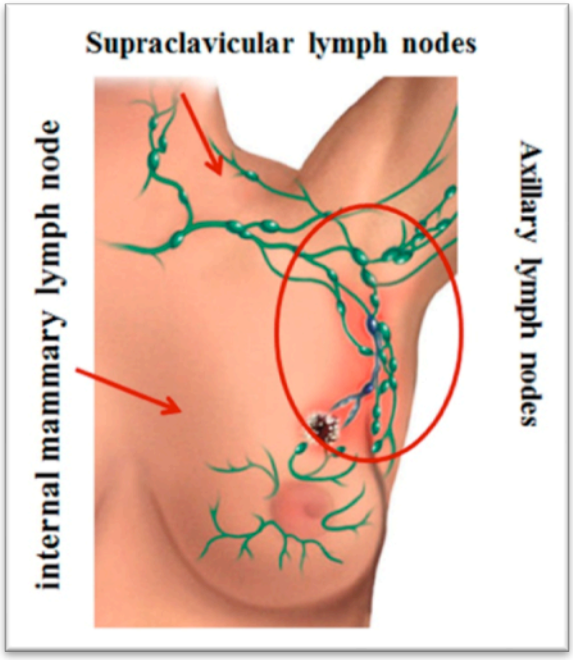

Figure 1. Lymphatic system that filters the lymph fluid draining away from the breast area. The armpit, or "axilla" is the main lymph node area involved in breast cancer and two secondary lymph node areas are the internal mammary and supraclavicular regions.

(Modified picture from http://doctorstock.photoshelter.com/image/I0000u4cGX0VP_Do)

Since the lymphatic system has a direct dispatch to spread primary tumor cells to the lymph nodes in breast cancer, primary tumor signatures have been considered as surrogates for lymph node metastasis. However, this persuasion has recently been controversial particularly in the context of transferring DNA methylation patterns from primary tumors to metastatic in breast cancer[19-21]. Considering that tumor dissemination is an early event in breast cancer [22], genetic and epigenetic analysis of tumors and metastatic lesions could provide results for biomarker discovery and may improve diagnosis, prognosis and proper management of treatment regimens in breast cancer patients.

1.2 Breast neoplasm pathophysiology

1.2.1 Hereditary breast cancer

Breast cancer is a heterogeneous disease with sequential accumulation of molecular, genetic and epigenetic alterations. The majority of breast cancers are sporadic; only a small

proportion of cases are susceptible of developing the disease by positive family history. Approximately 5-10% of cases are detected with *BRCA1* and *BRCA2* mutations. These two genes are conferred as high penetrance genes. Other high penetrance genes such as *TP53* and *PTEN* are detected at lower frequencies in hereditary breast cancer [23-25].

Over the last decade, it was increasingly demonstrated that harboring a germline mutation in *BRAC1/2* genes increases the risk of breast cancer by 60-80% [26, 27]. *TP53* mutations are the cause of a rare autosomal dominant cancer predisposition referred to as Li–Fraumeni syndrome (LFS). LFS has approximately 1% prevalence among the hereditary breast cancer syndromes[28]. The other type of familial breast cancer syndrome, with less than 1% frequency, is known as Cowden syndrome (CS), which is associated with mutations in *PTEN* gene [29, 30]. Along with *BRAC1/2*, *P53* and *PTEN* mutations, *CHEK2*, *ATM*, *BRIP1*, and *PALB2* were also identified as high frequency and low penetrance genes that are associated with the increased risk of breast cancer[31-33]. However, it was reported that more than 90% of breast cancers are independent of germline mutations[34] and are primarily due to either somatic genetic or epigenetic alterations.

1.2.2 Altered genes in sporadic breast cancer

Genetic and epigenetic alterations on genes can change the quality (structure) or quantity (expression) of gene products as well as impairing specific cellular pathways that encouraged paying particular attention to them for identification of responsible alterations leading to tumorigenesis.

Several studies on breast cancer cohorts have demonstrated the amplification and overexpression of several oncogenes such as: *ERBB2, HER2 /neu*, *CCND1*, *MYC*, fibroblastic growth factors (FGFs) and their receptors, *BCL2*, *CCNE*, *hTERT* and *H-ras* [35-42].

In addition to the amplification and overexpression of oncogenes, inactivation of tumor suppressor genes (TSGs) has been identified in breast tumorigenesis. Various mechanisms have been proposed for TSGs inactivation; point mutation of one allele and the LOH of the other allele are the most common cause of TSGs inactivation [43]. The second mechanism involved in the loss or reduction of TSGs expression is DNA hypermethylation of CpG islands located in promoter region of TSGs[22] (section1.5.2.2). Tumor suppressor gene downregulation by the

aforementioned mechanism have been reported in sporadic breast cancer such as *TP53*, *ATM*, *RB*, *BRCA1/2*, *CDH1*, *Cdksi* (*KIP*, *INK4*), *PTEN*, *ESR1* and *IGFs* [44]. Among the above tumor suppressor genes, *TP53* and its signaling pathway have a central role in different cellular responses and have a potential role in cancer development. Loss of p53 function and impaired p53 signaling pathway have been reported in many types of cancer[45].

1.3 *TP53* Tumor suppressor gene and protein structure

The *TP53* gene, located at 17p13.1, encodes the p53 protein, which has numerous function and protein-protein interactions. P53 has nine different isoforms originated from two distinct transcriptional start points as well as several alternative splicing patterns[46]. Studies on breast cancer cohorts indicated a correlation between mutation type and the clinical outcome of patients[47]. Therefore structure-function studies on wild type protein p53 are essential to understand functional consequences of mutations.

Human p53 protein is 393-aa in length, which contains three domains flanked by proline-rich linker regions[48]. P53 domains consists of a transactivation domain (N-terminal region, residues1–62), a sequence-specific DNA binding domain (core region, residues 94–292) and a C-terminal region that includes a tetramerization domain (residues 325–356) and a negative regulatory (residues 361–393) domain (Fig.1) [49].

1.3.1 The transactivation domain

The transactivation domain and its adjacent proline-rich region can activate p53 transcription and regulate the cellular levels of p53, as well as, regulate p53 function and stability. Phosphorylation of the N-terminus can affect the stability of p53. This domain interacts with p53 regulators such as MDM2, a ubiquitin protein, that targets p53 for degradation and acetyltransferases, p300 and CBP, which regulate p53 function by acetylation of the C-terminal region [51-53].

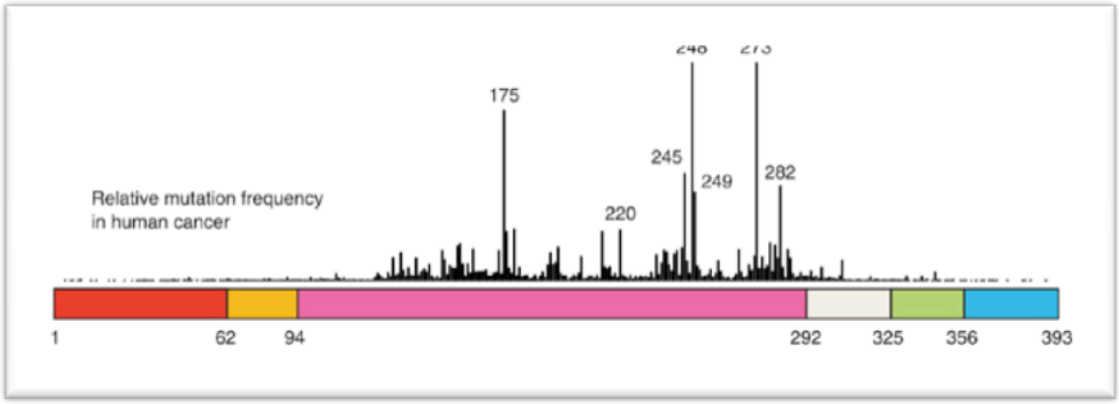

Figure 2. "Schematic view of the domain structure of p53. The columns indicate the relative frequency of cancer-associated mutations for each residue, data from the *TP53* mutation database of the International Agency for Research on Cancer (version R10, www-p53.iarc.fr)[49, 50]"

1.3.2 The DNA binding domain

The core region binds to the double stranded DNA by recognizing the sequence 'half-site' motifs 5'-Pu·Pu·Pu·C·(A/T)·(T/A)·G·Py·Py·Py-3' (Pu=A/G, Py=T/C) which is separated by up to 13 base pairs [54]. P53 regulates the cellular responses by stimulating transcription of many target genes in a selective manner. The affinity of binding p53 to its response elements is variable and depends on the recognition of the sequence. For example, this affinity is high for the genes involved in cell cycle arrest while it is low for the genes involved in apoptosis[55, 56].

1.3.3 The C-terminal domain

The tetramerization domain regulates the oligomerization state of p53. It has been suggested that nuclear p53 levels might be controlled by its oligomerization state[57]. The negative regulatory domain modulates p53 sequence-specific DNA binding by the core domain [22]. Post-translational modifications of the C-terminus in response to cellular stress can activate p53 [58] . However, recent evidence have shown that p53 with an unmodified C-terminus can actively bind to double-stranded DNA[59].

1.4 Normal p53 functions

P53 is a transcription factor with essential roles in diverse biologic activities. P53 is activated in response to cellular stress, which is followed by targeting various genes and microRNAs and activating different pathways including cell cycle arrest, apoptosis, DNA repair and senescence. Activation of either of these pathways depends on the nature of the input stress signals that are interpreted by the upstream mediators of p53. For example, DNA double strand breaks or gamma radiation activates ataxia-telangectasia mutated (ATM) kinase and CHK2 kinase, both kinases can phosphorylate p53. In addition, expression of oncogenes activate $P14^{ARF}$ that inhibits p53 degradation and increases levels of p53 protein [60].

1.4.1 Down-stream events of the p53 Pathway

1.4.1.1 Cell cycle arrest

The activated p53 networks can inhibit cell cycle at G1 or G2 phases. Upregulation of p21, p53 target gene, inhibits cyclin dependent kinase which acts upon G1 to S phase in the cell cycle. On the other hand overexpression of 14-3-3 sigma (σ), a protein binds to CDC25C, contributes in blocking the G2 phase of the cell cycle[61].

1.4.1.2 Apoptosis

The process of programed cell death can be regulated by different types of apoptotic pathways including the p53 pathway. Activated p53 induces expression of pro-apoptotic proteins such as Bax, Apaf1 and repressing expression levels of the anti-apoptotic proteins, Bcl-2 and Bcl-XL or survivin [62, 63]. Indeed, translocation of p53 to the mitochondria enables interaction with Bcl-XL, which results in cytochorom c release to the cytoplasm [64].

1.4.1.3 DNA repair

DNA damage can activate various repair mechanisms in cooperation with p53 via a post-translational modification dependent manner [65]. P53 modified proteins eventually elaborates a set of proteins, e.g. p48 and p53R2 (ribonucleotid reductase gene), to contribute in the pathway of

DNA repair [45]. Recent evidence have indicated that p53 modulate transcriptional activity of nucleotide excision repair (NER) genes which promote the global genomic repair [66]. Moreover, a potential role for p53 in base excision repair (BER) has been described, as BER deficiency in mutant p53 cells are found [67].

1.4.1.4 Mitochondrial mass and function

The integrity of mitochondrial function is necessary for various biosynthetic and bioenergetic pathways as well as the regulation of survival and death signals. Dysfunction and deregulation of mitochondria play an important role in the pathophysiology of cancers [68]. Additionally, variations in mitochondrial DNA (mt-DNA) copy number, the both increase and decrease, have been strongly reported in different type of cancers [69, 70].

In addition to its nuclear functions, p53 protein and its network have regulatory effect on mitochondria. Several genes encoding mitochondrial proteins are modulated by the transcriptional activity of p53 such as cytochorome-c oxidase 2 (SCO2), apoptosis-inducing factor (AIF), 16S rRNA and cytochorome-c oxidase (COX)[71-73]. Apart from regulatory role of p53 on mitochondrial proteins, it is also involved in mitochondrial biogenesis and copy number regulation through a direct interaction with mt-DNA polymerase gamma and mitochondrial transcription factor A (TFAM)[74-76].

Furthermore, p53 boosts mitochondrial genomic stability via stimulation of base excision repair[77, 78]. Human mitochondrial DNA mutation can be induced by generation of reactive oxygen species (ROS). An increase in mtDNA sensitivity to DNA-damaging oxidative stress, such as ROS, has been shown to be via deregulation of p53 protein in cancer cells[74, 79]. MtDNA has a higher mutation rate than nuclear DNA, due to its closeness to the respiratory chain, lack of histone proteins and limited DNA repair capacity. Most of the mtDNA mutations occur in the D-loop region, which is the major replication and transcription site of both the heavy and light strands of mtDNA[80, 81]. Previous studies have demonstrated that somatic mutation on the DNA-binding domain of *TP53* gene contributes to mtDNA mutation and mtDNA depletion which, indicates a possible role in breast cancer tumorigenesis [2, 82].

In contrast to the effects of p53 on mitochondria functional integrity, recent studies have revealed influences of mitochondrial dysfunction on p53 expression or function. These studies argued a potential functional decrease of p53 due to increases mitochondrial dysfunction according to Warburg effect in cancers[83-85]. Taken together, both P53 and mitochondria likely have reciprocal effect on each other, which may play an important role in pathogenesis of cancers.

1.4.1.5 Senescence and telomere maintenance

Senescence is the irreversible arrest of cell replication potency that occurs in response to different cellular stresses such as, unrepaired nuclear DNA damage or uncapped telomeres [86]. Dysfunction of p53 (and its pathway) contributes to bypasses the senescence checkpoints which leads to telomere dysfunction in cancer[87]. Telomeres are specific repeat sequences $(TTAGGG)_n$ located at chromosome ends. They have a key role in the maintenance of chromosomal stability [88]. The TTAGGG repeats shorten with each cell division because of end replication mispairing, oxidative damage and other end processing events[89, 90]. Tumor cells have extremely short telomeres in association with increased genomic instability[91, 92]. This suggests that telomere changes are implicated in cancer development.

Regulation of p16/Rb and p53/p21 pathways are important proliferation control mechanisms that are linked to telomere shortening in human cells[93] . Inactivation of p53 enables continued proliferation of cells with dysfunctional telomeres, ultimately promoting chromosomal instability and transformation [94, 95] whereas expression of p16 inhibits phosphorylation of retinoblastoma protein (pRB), thus preventing cell-cycle progression [96]. Recent data suggest that the severe genome instability present during telomere crisis, promotes secondary genetic changes that facilitate carcinogenesis[97, 98]. Several studies have also indicated that the up-regulation of p16 in senescent cells may be a consequence of telomere dysfunction [99] . This up-regulation of p16 has been suggested to compensate p53 deficiency in cells and may serve as a target in cancer therapies [100].

1.5 Mechanism of p53 inactivation in cancer

1.5.1 *TP53* mutation

The *TP53* gene is often mutated in a vast majority of human cancers (over 50% of cancers) and most of these mutations are detected on the DNA binding domain[60]. The frequency of *TP53* mutation outside the DNA binding domain is about 10%[101] (Figure.2). *TP53* mutations detected on the core region have been classified into two groups, I) replacing residues that contact DNA II) replacing residues responsible for DNA binding domain stabilization[102]. The DNA binding domain is more susceptible to mutation due to the low melting temperature of this domain. It means every substitution in this part can influence protein folding[103]. In addition to the melting temperature, a high frequency of CG rich dinucleotides located on the DNA binding domain could contribute to the high frequency of mutation in this domain [104].

TP53 mutations are mainly missense mutation that could lead to new oncogenic functions or a gain of function (GOF). Many cancer studies have reported accumulation of GOF mutant p53 at a higher level in advanced stages of cancer. It is believe that this accumulation may promote a greater interaction with a larger subset of DNA motifs and proteins by the mutant p53 proteins compared to the wild type counterpart. The interaction of the mutant p53 protein is depended on its newly gained structure and affinities to DNA sequence and proteins [105].

The frequency of *TP53* mutations in breast cancer is approximately 20% and the frequencies are lower in node-negative patients (15-18%) than in node positive patients[106, 107]. The prevalence of p53 mutations in breast cancer patients is lower than that of other cancers[62, 106] (Table. 2).

Table 2. Frequency of p53 mutations in human cancers.

Cancer	Number of cancer	Number of Mutant	Frequency
Breast carcinoma	14389	3390	23.56
Colorectal carcinoma	9183	3967	43.2
Lung (NSCLC)	6126	2314	37.77
Head and Neck SCC	5654	2185	38.65
Ovarian carcinoma	4401	1997	45.38
Bladder carcinoma	4271	1156	27.07
Hepatocellular carcinoma	3327	945	28.4
Glioblastoma	2706	633	23.39
Gastric carcinoma	2467	739	29.96
Esophageal SCC	2444	1136	46.48
Prostate ca.	1395	205	14.7
Astrocytoma	1247	451	36.17
B-Chronic Lymphocytic Leukemia	1200	144	12
Pancreatic cancer	1107	342	30.89
Acute Myelogenous Leukemia	852	78	9.15
Osteosarcoma	816	158	19.36
Soft Tissue Sarcomas	791	73	9.23
Cervical Cancer	766	44	5.74
Endometrial tumor	724	184	25.41
Non-Hodgkin's Lymphomas	708	137	19.35
B-cell Lymphoma	638	101	15.83
Sarcoma	627	36	5.74
Thyroid Carcinoma	603	58	9.62
Brain Tumor	599	155	25.88
B-Acute Lymphoblastic Leukemia	564	77	13.65
Renal cell ca.	528	70	13.26
Gliomas	481	112	23.28
Esophageal ADC	424	248	58.49
Myelodysplastic synd.	422	29	6.87
B-Lineage Diffuse Large Cell L	357	59	16.53
Adult T-cell Leukemia	328	54	16.46
Skin Squamous Cell Carcinoma	312	132	42.31
Lung (SCLC)	288	148	51.39
Basal Cell Carcinoma	284	124	43.66
Follicular lymphoma	274	27	9.85
Rectal ca.	261	116	44.44
Urothelial TCC	260	111	42.69
Esophageal ADC (Barrett)	253	77	30.43
Colorectal adenoma	238	14	5.88
Nasopharyngeal carcinoma	232	34	14.66
Melanoma	210	54	25.71
Multiple Myeloma	209	20	9.57
Uterine cancer	189	71	37.57
Wilm's tumor	177	21	11.86
Hepatic angiosarcomas	169	5	2.96
Neuroblastoma	147	4	2.72
Mantle Cell Lymphoma	143	23	16.08
Oligodendrioglioma	143	28	19.58
Urothelial ca.	143	73	51.05
Colorectal Carc. Metast.	142	77	54.23

Data reproduced from the 2008R2 release of the UMD database (available online; http://p53.free.fr)

1.5.2 Molecular mechanism of p53 inactivation

It has been estimated that dysfunction of the p53 pathway contributes to over 80% of human cancers [108]. The p53 pathway consists of hundreds of genes, involved in different biological outcomes. They interact with other signaling pathways and act as autoregulatory feedback loops following p53 activation [61, 109]. P53 protein expression and function is tightly regulated by its autoregulatory feedback loops. In addition to regulatory loops impairing p53 function and its downstream targets, epigenetic modifications and aberrant microRNA expression (targeting p53 pathway components) in cancer are two molecular mechanisms presumed to impair the p53 pathway.

1.5.2.1 Changes in p53 auto-regulatory feedback loops

The p53 feedback loops consist of proteins that are activated or expressed by p53 activation that contribute to alter p53 expression level and activity. A variety of studies revealed seven negative feedback loops that down-regulate p53 activity. Three positive feedback loops have been detected that increase p53 activity. The core regulatory feedback loop is known as p53/MDM2/ $p14^{ARF}$ [45] (Figure 3.).

Mdm2 inactivates the tumor suppressor p53 in response to stress by regulation the duration of p53 activity. Mdm2 binds directly to p53 to inhibit transcription and export p53 from the nucleolus to the cytoplasm, resulting in the degradation of p53 by ubiquitination [110, 111]. The role of $p14^{ARF}$ in the p53 pathway is to indirectly regulate the level of p53 protein. The $p14^{ARF}$ protein inhibits degradation of p53 protein by keeping Mdm2 in the nucleolus and by restricting E3 ubiquitin protein[111].

The other important positive regulatory loop is PTEN/AKT that modulates p53 activity (Figure 3.). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is an essential component of the *TP53* gene response upon DNA damage. Pten protein indirectly regulates p53 function through keeping Akt inactive and making Mdm2 incapable of translocating into the cell nucleus for degradation of p53[112, 113]. More importantly, Pten prevents p53 degradation in the presence of Mdm2 by increasing p53-mediated transcription [112]as well as binding to p53, leading to an increase in the half-life of p53[114].

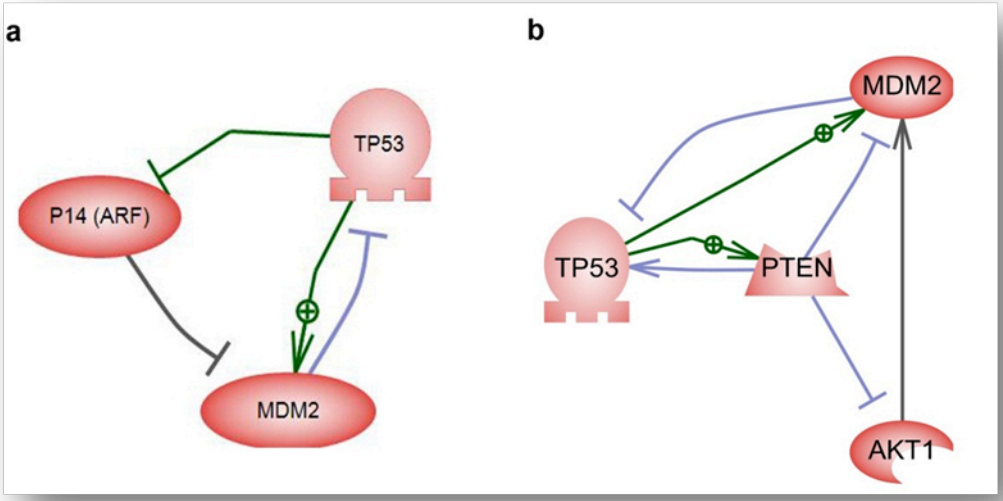

Figure 3. P53 feedback loops. a) Core regulatory $P14^{ARF}$ / MDM2 loop, b) PTEN/AKT/ MDM2 loop.

The *P14ARF*/*MDM2*/*TP53* and *PTEN* pathways are the two critical *TP53* control pathways, and alteration of either of these pathways can induce a variety of cancers and they are thus the focus of many breast cancer investigations[115-120].

1.5.2.2 Epigenetic Alterations

Epigenetic alterations are recently recognized as one of the important features of human cancers. DNA methylation and covalent histone modifications are two major epigenetic modifications[121, 122].

DNA methylation of the cytosine located 5′ to a guanosine is one of the most important modifications of genomic DNA in eukaryotic cells. Methylation of cytosine at CpG dinucleotides is described as an epigenetic regulatory mechanism of genomic function that plays an important role in different biological processes including embryogenesis [1], genomic imprinting [2], X-chromosome inactivation, and cancer [3, 4]. DNA hypermethylation has been suggested as an alternative mechanism to gene mutation or deletion that leads to loss of genes

function. This alteration has been known as epimutaion due to phenotypic similarities to genetic mutations[123].

The contribution of aberrant DNA hypermethylation of the transcriptional silencing and carcinogenesis of cancer related genes has been demonstrated in different cancer types[22, 124]. The methylation profile of genes involved in critical molecular processes such as cell cycle control, DNA repair and angiogenesis in breast cancer has been investigated[20, 21, 125, 126]. Promoter hypermethylation of the p53 pathway in cancers with low frequency of *TP53* mutations have been identified and suggested that malfunction of p53 can be mediated via an epigenetic mechanism[70, 127].

1.5.2.3 Aberrant microRNA expression

miRNAs are a class of small non-coding regulatory RNA, which is synthesized by RNA polymerase II. Mature miRNAs, 19-25 nucleotide in length are associate with RNA-induced silencing complex (RISC) and bind to the complementary protein coding messenger RNA (mRNA) target that eventually mediates gene expression modulation [128]. Several reports have demonstrated important roles for miRNA in different biological processes. Recently aberrant expression of miRNAs has been indicated to the pathogenesis of different human cancers [129-131]. These deregulated miRNAs contribute functionally to the initiation and progression of cancer by altering the expression of crucial pathways involved in cell proliferation and survival[132, 133].

Numerous studies have been directed toward understanding the cross talk between *P53* transcription regulation and miRNAs. The impact of miRNAs in the dysregulation of p53 and its signaling pathway in solid tumors, however, is not well characterized [108, 134].

1.6 Biomarkers

Biomarkers are substances found in biological fluid or tissues, which are differentially in disease and normal states. Biomarkers are typically associated to pathogenic processes or to pharmacologic responses following treatment[135] . These can be indicated as single nucleotide polymorphisms (SNPs), DNA methylation, changes in mRNAs or miRNAs, protein and metabolite levels. Cancer biomarkers can be divided into three categories[136] : (a) Diagnostic (classification) biomarkers are used to detect and identify a given type of cancer in an individual. This type of biomarker is expected to have high levels of diagnostic sensitivity and specificity. (b) Prognostic biomarkers are commonly used once the disease status has been established. (c) Stratification (predictive) biomarkers can serve to predict the likely response to a drug before starting treatment, thus classifying individuals as responders or non-responders[16].

The candidate biomarkers should have very high specificity and sensitivity (close to 100%) to be accepted for clinical utility. In addition, they need to be economical, easy to measure and reproducible by different methods. So far only a few biomarkers have been approved by the Food and Drug Administration (FDA) [69]. Moreover, there are remarkable limitations for the implementation of the huge number of biomarker candidates represented in the majority of reports. These limitations mainly lie in the lack of technical validation, the low number of patient and control subjects in a study, and the inability to select a cohort with the same clinicopathological characteristics.

The most important biomarkers for breast cancer prognosis and therapy are Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth factor Receptor (HER2), Cancer-Antigen 15/3 (CA-15-3) or Cancer-Antigen (CA-27-29), Cytokeratins and Oncotype DX [137]. Considerable progress has recently been made in exploring components of the pathways linked to tumorigenesis. Increasing our knowledge of specific biologic pathways related to the initiation and progression of cancer can lead to the development of novel biomarkers for early diagnosis and improvement of therapeutic regimens for cancer patients.

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2. AIM OF THE STUDY

Mechanism of p53 inactivation independent of *TP53* mutations in breast cancer leading to biomarker discovery

The frequency of *TP53* mutations in breast cancer is lower than that of many other cancers. Other than *TP53* mutation, alteration of upstream and/or downstream *TP53* regulators (autoregulatory loops, proteins and miRNAs) is another potential mechanism, which could modulate the inactivation and suppression of this gene and its signaling pathway in breast cancer. These alterations might provide further clue to establish meaningful biomarkers for breast cancer patients. Therefore, to find the molecular mechanism involved in tumor suppressor p53 protein deregulation independent of mutation: First, we assessed the aberrant methylation of CpG islands of genes involved in two autoregulatory feedback loops ($P14^{ARF}$/*MDM2/TP53* and *PTEN*) in breast cancer patients, as well as evaluating each hypermethylated CpG sites to the recognition sites of the transcription factors along the promoter region. The possibility of using hypermethylated genes in serum samples as a blood-based test fro breast cancer was explored. Second, considering aberrant DNA hypermethylation is a reversible alteration, on the basis of demethylation therapy and *in-silico* pathway analysis the over-all proteomic characterization of breast neoplasm in the presence or lack of *TP53* mutation were identified. Third, aberrant expression of miRNAs impairing the p53 pathway depending on p53 status in breast cancer has been characterized.

The Relationship between aberrant DNA methylation of p53 related pathways and downstream events

Activated p53 targets many genes and regulates several biologic processes including telomere and mitochondria maintenance. A certain telomere length and normal mitochondrial function are necessary for genomic stability and cellular integrity. Thus, shortened telomere length and mitochondria dysfunctions are the focus of many cancer investigations and are suggested to play an important role in the pathophysiology of cancers.

Taken together, these can provide new insights into epigenetic silencing of some of the regulatory pathways, leading to the deregulation of p53 protein, which most probably influences on the telomere length shortening and on the mtDNA vulnerability in breast cancer. In this context, we investigated a potential link between promoter hypermethylation of the *TP53*/*P21* and *P16/Rb* genes and telomere length shortening. The correlation of aberrant methylation patterns of $P14^{ARF}$*/MDM2/TP53/PTEN* and mtDNA alteration, including D-loop region integrity and mtDNA content were also studied. Lastly, the study was extended to understand the link between the p53 pathway and epigenetic alterations by treating breast cancer cell lines with a demethylating agent, 5-aza-2′-deoxycytidine. Using LC-MS-MS analysis, proteins influenced by alteration in DNA methylation status were identified. Additionally, a comprehensive *in-silico* pathway analysis was performed to characterize biological responses that allowed us to detect aberrant proteins expression involved in mitochondrial function and in telomere maintenance in breast neoplasm.

Contributions of aberrant DNA methylation signature of primary tumor to metastatic lesion

The aberrant hypermethylation of genes involved in critical molecular processes e.g. cell cycle control, DNA repair and angiogenesis in primary breast cancer has been a center of attention in many studies. However, the DNA methylation profile of metastatic tumors in breast cancer patients is less characterized and poorly understood. In a previous study of our group, methylation signatures of 42,528 CpG sites from 22 breast cancer candidate genes demonstrated promoter hypermethylation in 10 genes. Our assessment of the DNA methylation status of genes involved in two p53 autoregulatory feedback loops revealed hypermethylation of two genes in those loops. Here, we further analyzed the contribution of significant aberrant methylation profile of twelve cancer related genes from the aforementioned studies (*APC, BIN, BMP6, BRCA1, CST6, ESR-b, GSTP1, P14, P16, P21, PTEN* and *TIMP3)* in metastatic axillary lymph nodes in comparison to the primary tumor tissues and the adjacent normal tissues from the same breast cancer patients to identify a potential metastatic signature of the candidate genes.

3. PEER-REVIEW AND UNDER -REVIEW MANUSCRIPTS

3.1. Published review article

Specificity of methylation assays in cancer research: a guideline for designing primers and probes

Authors: Zeinab Barekati, Ramin Radpour, Corina Kohler and Xiao Yan Zhong

Journal: Obstetrics and Gynecology International. 2010, pii: 870865

Summary: Technically for applying DNA methylation alterations as a biomarker, certain specificity and sensitivity needed to be defined. This review focuses on important criteria for designing specific primer pairs and probe to avoid false positive detection of DNA methylation profiles. Indeed, some information about related online web tools is provided.

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Review Article

Specificity of Methylation Assays in Cancer Research: A Guideline for Designing Primers and Probes

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DNA methylation is an epigenetic regulation mechanism of genomic function, and aberrant methylation pattern has been found to be a common event in many diseases and human cancers. A large number of cancer studies have been focused on identification of methylation changes as biomarkers (i.e., breast cancer). However, still clinical use of them is very limited because of lack of specificity and sensitivity for diagnostic test. This highlights the critical need for specific primer and probe design to avoid false-positive detection of methylation profiling. The guideline and online web tools that are introduced in this paper might help to perform a successful experiment and to develop specific diagnosis biomarkers by designing right primer pair and probe prior to experimental step.

1. Introduction

DNA methylation of cytosine located 5′ to a guanosine is one of the most important modifications of genomic DNA in eukaryotic cells. Methylation of cytosine at CpG dinucleotides is described as an epigenetic regulation mechanism of genomic function that plays an important role in different biological processes including embryogenesis [1], genomic imprinting [2], X-chromosome inactivation, and cancer [3, 4].

Aberrant methylation pattern has been found to be a common event in many cancers [5–7]. Global hypomethylation is considered to play a role in carcinogenesis; however, local hypermethylation changes gene expression [8]. This hypermethylation alteration resulted in transcriptional inactivation followed by silencing of promoter at nearby tumor suppressor genes, contributing to development of cancer. The hypermethylation was thought to be an early event in carcinogenesis [9–12]. A large number of studies in cancers including breast cancer have focused on the use of CpG island hypermethylation profiling as cancer biomarkers in tissue and circulating cell-free DNA of patients, with the aim of improving cancer treatment via accurate early diagnosis, noninvasive diagnosis, prognosis, and prognosis therapy selection [7, 13–18].

Recent technology development has provided the analysis of DNA methylation in a genome-wide scale [19, 20] which may not be easily accessible for many institutions. Thereby, in most of the research centers methylation assays can be only determined on gene-by-gene-based methods that use bisulfite conversion. The bisulfite reaction was first described in early 1970s [21, 22]. Since the first description of bisulfite reaction in the application of studying CpG sites, many methods based on the same principle have been developed and categorized according to primer designing strategies. Based on primer designing strategies two different DNA methylation assays are described, methylation-independent-specific PCR (MIP) primers and methylation-specific PCR (MSP) primers [23].

Primer and probe design for methylation assays based on bisulfite conversion is challenging because of the DNA composition after bisulfite modification. One of the most critical steps for methylation study is designing primers and probes for the modified DNA and it needs special constrains on primers or probe and their location on the DNA. A large number of studies have been focused on identification of

biomarkers; however, the clinical use of these biomarkers is still very limited because of lack of specificity and sensitivity for diagnostic test. This highlights the critical need for specific primer and probe design to avoid false-positive detection of methylation.

We review a brief guideline of CpG island prediction, designing primers and probes for MIP and MSP assays that are used for methylation studies based on bisulfite conversion. Some important web-tools for methylation studies are introduced as well.

2. CpG Island Prediction

Methylation at the cytosine bases of CpG dinucleotide-rich region mostly within 0.5–4 kb are known as CpG islands [24, 25]. Although analysis of the methylation status of some critical CpG sites as biomarkers are better than others, it is essential to find CpG islands at the promoter region of candidate genes which are in close proximity to the transcription start site.

In order to predict CpG islands as target region, the following rules should be applied.

(I) If CpG island prediction is used for primer design and more than one island is found, any of the predicted islands can be a target region for primer selection.

(II) If a CpG island size is smaller than the minimum product size, the primer pair should span the whole island.

(III) If a CpG island size is greater than the maximum product size, the primer pair should be within the island.

(IV) If a CpG island size is between the minimum and maximum product size, at least two thirds of the island region should be amplified.

3. Methylation-Independent-Specific PCR (MIP) Primers

MIP primers are used in different PCR-based methylation analysis methods including bisulfite-sequencing PCR (BSP) (in 1992, [26]), pyrosequencing [27, 28], combined bisulfite restriction analysis (COBRA) [29], methylation-sensitive single-nucleotide primer extension (MS-SnuPE) [30–32], methylation-sensitive melting curve analysis (MS-MCA) [33], methylation-sensitive high-resolution melting (MS-HRM) [34], matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with base-specific cleavage and primer extension [35, 36], heavy methyl [37], and microarray DNA methylation profiling technique based on bisulfite conversion, that is, methylation-specific oligonucleotide microarray (MSO) [38].

Incomplete bisulfite modification of DNA is sometimes a concern [39] and results in high representation of methylation levels in studied samples. Successful application of MIP methods depends on whether PCR primer could be designed to amplify the complete modified fragment of interest.

Table 1: The main characteristics for primer/probe designing in DNA methylation profiling techniques based on bisulfite conversion.

Primer/Probe	Main characteristics
MIP primer	(i) No CpG sites within the sequence. (ii) Including an adequate number of "C"s (no-CpG) in the sequence. (iii) Spanning a maximal number of CpG sites in the amplicon. (iv) Long length primer (25–30 mer). (v) Amplicon size maximum 500 bp.
MSP primer	(i) Containing as much CpG sites as possible especially at 3′-end of the primer. (ii) Considering the same CpG sites in the primer sequence for methylated DNA and unmethylated DAN primers. (iii) Similar Tm values for both the methylated DNA and unmethylated DAN primers. (iv) Amplicon size maximum 500 bp.
Probe	(i) Including CpG sites to maximize specificity. (ii) Including several "C"s (no-CpG) in the sequence. (iii) Probes length 15–30 mer. (iv) Amplicon size 50–150 bp (max 300 bp).

To reduce bias of bisulfite-modified DNA against unmodified or incompletely modified DNA or even unsuccessful experimental PCR optimization, primer pair should be picked from a region that have adequate number of cytosines "C"s (no-CpG) in the original sequence [40]. Primer pairs with more "C"s will be preferred by receiving higher weighing scores and increasing the annealing temperature (Table 1). Besides general consideration for designing primer pair, the following constraints are enforced for MIP primer design.

(I) Primers should not contain any CpG sites within their sequence to avoid discrimination against methylated or unmethylated DNA (Figure 1).

(II) Primers should have an adequate number of "C"s (no-CpG) in their sequence to amplify only bisulfite modified DNA. Primers with more "C"s will be preferred (at least 30%) [40] (Figure 1).

(III) A good primer pair should span a maximal number of CpG sites in the selected amplicon to map as many CpG sites as possible.

(IV) If CpG island prediction is not used for primer selection, selected amplicons must span at least 5 CpG sites as a default.

(V) Long length primer (25–30 mer) is preferred to ensure uniqueness of the primer [39].

(VI) Primer sets should not amplify more than 500 bp because DNA degradation occurs by bisulfite modification.

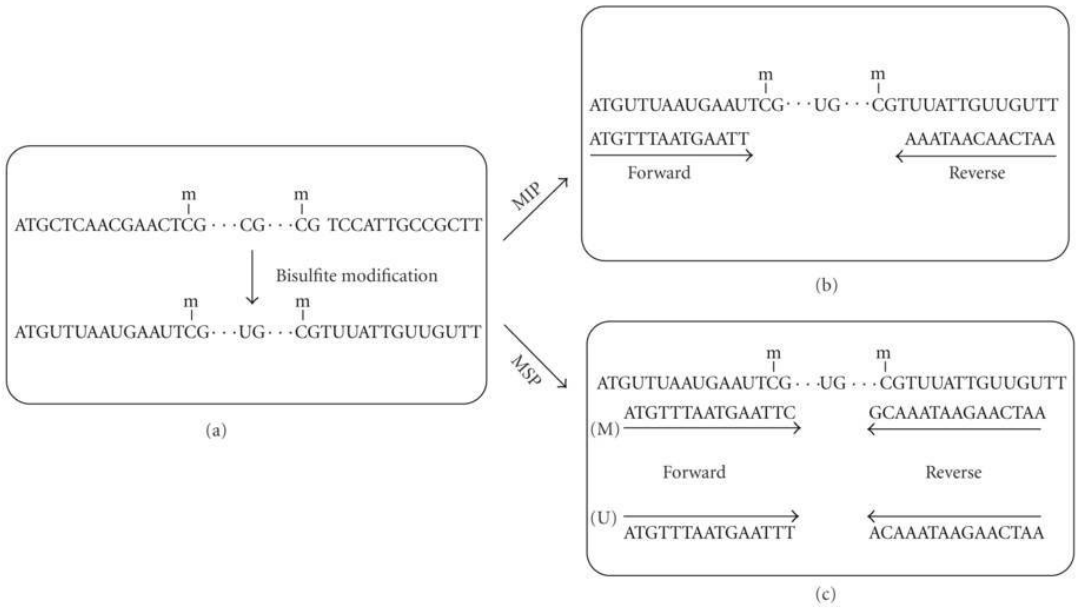

FIGURE 1: Primer design for DNA methylation profiling techniques based on bisulfite conversion. (a) First DNA is treated with sodium bisulfite to convert all unmethylated cytosines to uracil. To analyze DNA methylation status of the interest genes, converted DNA is amplified based on two different primer designing strategies: methylation-independent specific PCR (MIP) and methylation-specific PCR (MSP). (b) In MIP, DNA molecules are amplified using primer pairs containing cytosines (no-CpG) in their sequence. (c) In MSP, primer pairs are designed to specifically amplify either methylated (M) or unmethylated (U) DNA by containing CpG site in their sequence that makes possible to distinguish the methylated sequence from the unmethylated sequence.

4. Methylation-Specific PCR (MSP) Primers

Methods based on MSP primers are considered to have the highest analytical sensitivity and are designed to specifically amplify either methylated or unmethylated DNA by using primers that distinguish the methylated sequence from the unmethylated sequence [23, 40]. The precision and sensitivity of MSP depends on appropriate primer or probe design not prone to false-positive results [23]. MSP primers-based methods include methylation-specific PCR (MSP) [40], methylight [41, 42], SYBER green-based quantitative MSP [43, 44], sensitive melting analysis after real-time MSP (SMART-MSP) [45], and methylation-specific fluorescent amplicon generation (MS-FLAG) [46]. The specificity of methylation-based PCR methods is achieved by appropriate primer pair or probes design (Table 1). The following constraints are recommended to reduce false-priming events for amplification of methylated DNA.

(I) To discriminate between a methylated and unmethylated DNA fragment, primers have to contain as much CpG sites as possible (at least one CpG) preferably at the very 3′-end. At least one of the last three bases at 3′-end of the primer has to be a CpG "C" (Figure 1).

(II) A part from CpG site(s) at the very 3′-end, additional CpG sites in a primer sequence is preferred (Figure 1).

(III) Primers for methylated DNA and unmethylated DNA should contain the same CpG sites in their sequence. For example, a forward primer for methylated pair has this sequence: ATAAGTATT CGTTAATGGTTCGA, the forward primer in the unmethylated pair must also contain the two CpG sites, for example, ATAAGTATTTGTTAATGGTT TGA. But they may differ in length and start position [3].

(IV) The two sets of primers for methylated and unmethylated DNA should have similar T_m values (max T_m difference 5°C).

(V) Elimination of secondary structure formation and primer-dimer pairs by increasing primer length.

(VI) Primer sets should not amplify more than 500 bp because DNA degradation occurs by bisulfite modification.

False-priming event can be prevented by designing appropriate primers and increasing annealing temperature. Having an appropriate negative control in the experiment might help to find out false-priming events.

5. Guidelines for Probe Designing

In methylation studies, the discrimination between methylated and unmethylated DNA is achieved by three ways:

design of primers that contain or does not contain CpG sites, design of fluorescent labeled probe (for instance MSO and bead array), and design of the both primer and probe, that is, methylight technology [41]. MIP and MSP methods are associated with false positive results. By using fluorescent probes, for instance methylight methodology or applying heavy methyl probe-based methodology, the false positives can be limited. Using probe as a detection method increases the specificity to discriminate between methylated and unmethylated DNA by designing probes that contain additional CpG sites [40]. The selection of new primer pairs for methylation-specific PCR and suitable hybridization probes for real-time PCR-based assays require the identification of the CpG sites that are methylated (Table 1). Moreover, using probe provides possibility to detect more than one target with multiplex reaction by different reporter dyes [38, 47].

In addition probe-based assays can provide quantitative information; further advantages are the speed and high throughput of the 96-well-based, real-time PCR system and the omission of all postamplification steps, which has less labour and the risk of contamination. Also, the efficiency of individual reactions is accessible from the slope of the amplification plot in the logarithmic phase. This allows for the direct quality control of every amplification reaction and the identification of samples containing impurities or poor template that interfered with optimal amplification and thereby with the quantification [48].

A general guideline for probe designing is described as follows.

(I) The probe sequences should include 3 to 5 potential methylation sites to maximize specificity and reduce false-priming event.

(II) The probe binding sites should include several cytosines in the original sequence to ensure specificity for converted DNA and overcome false positives due to incomplete bisulfite conversion.

(III) Long repetitive stretches should be avoided.

(IV) Probe Tm value should be 10°C higher than primers.

(V) G + C content should be 30%–80%.

(VI) No G should be at the 5′ end.

(VII) Probes should have 15–30 mer in length.

(VIII) No more than two G + C should be at the 3′ end.

(IX) Amplicon size should be 50–150 bp (max 300 bp). The PCR products should be as short as possible, to maximize efficiency (especially important for the analysis of fragmented DNA isolated from formalin-fixed, paraffin-embedded biopsies, and circulating cell-free DNA).

6. Online Web Tools for Methylation Study

6.1. DNA Methylation Analysis Databases

(i) Entrez Gene: (http://www.ncbi.nih.gov/entrez).

(ii) GDB: Human Genome Database (http://www.gdb.org/).

(iii) DNA methylation database: public resource to store and standardise DNA methylation data (http://www.methdb.de/).

(iv) methBLAST: similarity search program designed to explore in silico bisulfite modified DNA, either or not methylated at its CpG dinucleotides (http://medgen.ugent.be/methBLAST/).

(v) DNA Methylation Society: an international scientific society open to all those interested in any aspects of biological methylation (http://www.dnamethylation.net/).

6.2. Promoter Prediction Tools

(i) FirstEF: first-exon and promoter prediction program for human DNA (http://rulai.cshl.org/tools/FirstEF/).

(ii) Promoter 2.0 Prediction Server: Promoter 2.0 predicts transcription start sites of vertebrate PolII promoters in DNA sequences (http://www.cbs.dtu.dk/services/Promoter/).

(iii) WWW Promoter Scan: predicts Promoter regions based on scoring homologies with putative eukaryotic Pol II promoter sequences (http://thr.cit.nih.gov/molbio/proscan/).

(iv) McPromoter MM: The Markov Chain Promoter Prediction Server. McPromoter is a program aiming at the exact localization of eukaryotic RNA polymerase II transcription start sites (http://genes.mit.edu/McPromoter.html).

6.3. CpG Island Prediction Tools

(i) CpG Island Searcher (http://cpgislands.usc.edu/).

(ii) CpG Plot (http://www.ebi.ac.uk/emboss/cpgplot/).

(iii) MethPrimer (http://www.urogene.org/methprimer/).

(iv) CpGProD (CpG Island Promoter Detection): CpGProD is a mammalian-specific software which proposes to identify the promoter regions associated with CpG islands (CGIs). CpGProD uses the structural characteristics of the CGIs associated with promoters (start CGIs). In the first step, CpGProD searches for all the CGIs located over the sequences and, in the second step, CpGProD identifies start CGIs and orientation of the potential promoters (http://pbil.univ-lyon1.fr/software/).

(v) CpG island Explorer for local installation (http://www.hku.hk/).

6.4. Methylation PCR Primer Design Tools

(i) MethPrimer: CpG island prediction, MSP, MSI primer design. By using this software 5′ and 3′ ends of primer pair should have sites where conversion has occurred (C to T). This is to avoid amplification bias towards the unconverted sequence (http://www.urogene.org/methprimer/).

(ii) BiSearch: BSP and MSP primer design (http://bisearch.enzim.hu/).

(iii) PerlPrimer: PerlPrimer is a free, open-source application written in Perl that designs primers for standard PCR, bisulfite PCR, real-time PCR (QPCR) and sequencing. It aims to automate and simplify the process of primer designing (http://perlprimer.sourceforge.net/).

(iv) BiQ Analyzer: software tool for easy visualization and quality control of DNA methylation data from bisulfite sequencing (http://biq-analyzer.bioinf.mpi-inf.mpg.de/).

6.5. Methylation BLAST (metthBLAST). methBLAST (http://medgen.ugent.be/methBLAST/) is a sequence similarity search program designed to explore *in silico* bisulfite modified DNA (either or not methylated at its CpG dinucleotides) to provide a search portal for validated methylation assays. The tool is mainly developed to find primer binding sites and hence addresses specificity for PCR-based assays that use bisulfite converted DNA as input material, including bisulfite sequencing, methylation-specific PCR, COBRA, bisulfite-PCR-SCCP (BiPS), Ms-SNuPE, and PCR melting curve analysis.

7. Discussion

The large number of investigations such as human epigenome project (HEP) and cancer studies focused on DNA methylation analysis based on bisulfite modification provided valuable information about methylation variable positions that might influence genes activity (http://www.epigenome.org) [7, 16, 49, 50]. Increasing knowledge about methylation status of genes involved in carcinogenesis can lead to discovering new biomarkers that could be used for early detection, management, diagnosis or therapeutic approaches in cancer patients. Developing biomarkers by methylation analyzing methods requires accuracy, sensitivity, low-false-positive and false-negative rates and high-throughput evaluation of single CpG sites. Although different useful technologies exist for methylation assessment, no method is universal. While besides choosing a method according to type of samples and possessed laboratory special equipment, right choice of CpG island and primer or probe will minimize the risk of failed experiment.

Right primer and probe design is crucial for successful PCR amplification of bisulfite-modified DNA. Bisulfite reaction not only causes the expected conversion of cytosines to uracils, but also causes undesired DNA strand breakage. Loss of DNA during the subsequent purification step is another concern especially when studying microdissected DNA samples. All these factors pose challenges to downstream PCR applications and primacy of designing primers and probe for such PCR-based assays. Mostly, amplification of a product size greater than 500 bp is difficult after bisulfite-modified DNA template; hence, it might be better to set the default product size range as 100–500 bp for primer design. Another option that differs from standard PCR is primer length. Bisulfite conversion-based PCRs generally require longer primers. Primers with a length of approximately 30 mer usually yield successful results [39]. The reason is that bisulfite modification decreases considerably GC content of DNA templates and produces long stretches of "T"s in the sequence that makes it difficult to pick primers with acceptable Tm values or stability. In other words, in order to discriminate modified DNA and unmodified or incompletely modified DNA, enough number of "C"s is required in primers and probes, which makes picking stable primers more demanding. Thus, to achieve better duplex stability, choosing longer primer is necessary as T_m of DNA. In practice, size of primers for such PCR-based assays usually ranges from 20 to 30 mer [3, 40, 51].

Much more effort is needed to validate an experiment for clinical use of biomarkers such as easy to use method, sensitivity and specificity, appropriate primers and probes, easily interpretable results, and cost-effectiveness. The guidelines and the online web tools that are introduced in this review might help to have a successful experiment and to develop specific diagnosis biomarkers by designing right primer pair and probe prior to experimental step.

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3.2 Published research article:

Correlation of telomere length shortening with promoter methylation profile of p16/Rb and p53/p21 pathways in breast cancer

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Summary: Relative telomere length and its potential correlation with methylation status of *TP53*, *P21* and *P16* promoters in breast cancer patients have been investigated. The results demonstrated a significant shortening of telomere regions in tumour tissues compared with adjacent normal tissues, which correlated with a different level of hypermethylation in the *TP53*, *P21* and *P16* promoters. Shortened telomere and promoter hypermethylation of related genes have potential to be served as breast cancer biomarkers.

Author contributions: Zeinab Barekati was involved in performing the experiment, data analysis and writing the manuscript **(* Equal contribution).**

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Correlation of telomere length shortening with promoter methylation profile of p16/Rb and p53/p21 pathways in breast cancer

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Unregulated cell growth, a major hallmark of cancer, is coupled with telomere shortening. Measurement of telomere length could provide important information on cell replication and proliferation state in cancer tissues. Telomere shortening and its potential correlation with downregulation of cell-cycle regulatory elements were studied by the examination of relative telomere length and methylation status of the *TP53*, *P21* and *P16* promoters in tissues from breast cancer patients. Telomere length was measured in 104 samples (52 tumors and paired adjacent normal breast tissues) by quantitative PCR. Methylation profile of selected genes was analyzed in all samples using a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS). Our results demonstrated a significant shortening of tumor telomere regions compared with paired adjacent normal tissues ($P<0.001$). Similarly, telomere lengths were significantly shorter in advanced stage cases and in those with higher histological grades ($P<0.05$). Telomere shortening in cancer tissues was correlated with a different level of hypermethylation in the *TP53*, *P21* and *P16* promoters ($r=-0.33$, $P=0.001$; $r=-0.70$, $P<0.0001$ and $r=-0.71$, $P<0.0001$, respectively). The results suggested that inactivation of p16/Rb and/or p53/p21 pathways by hypermethylation may be linked to critical telomere shortening, leading to genome instability and ultimately to malignant transformation. Thus, telomere shortening and promoter hypermethylation of related genes both might serve as breast cancer biomarkers.
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Keywords: telomere length; breast cancer; biomarker; DNA methylation; MALDI-TOF MS; quantitative real-time PCR

Telomeres are specific repeat sequences $(TTAGGG)_n$ located at chromosome ends. They have a key role in the maintenance of chromosomal stability.[1] The TTAGGG repeats shorten with each cell division because of end replication mispairing, oxidative damage and other end processing events.[2,3] Tumor cells have extremely short telomeres[4,5] in association with increased genomic instability.[6,7] This could suggest that telomere changes are implicated in cancer pathways. Similarly, Meeker *et al*[8] observed that telomere length abnormalities occur early in epithelial carcinogenesis. Therefore, telomere length may serve as a useful biomarker in human cancers.

Regulation of p16/Rb and p53/p21 pathways are important proliferation control mechanisms that are linked to telomere shortening in human cells.[9] Inactivation of p53 enables continued proliferation of cells with dysfunctional telomeres, ultimately promoting chromosomal instability and transformation,[10,11] whereas expression of p16 inhibits phosphorylation of the retinoblastoma protein (pRB), thus preventing cell-cycle progression.[12] Recent

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data suggest that the severe genome instability present during telomere crisis, promotes secondary genetic changes that facilitate carcinogenesis.[13,14] Other studies indicated that upregulation of *p16* in senescent cells may also be a consequence of telomere dysfunction.[15]

Optimal telomere length determination is achieved by real-time polymerase chain reaction (PCR).[16] This method relies on the use of telomere primers consisting of a six nucleotide repeated pattern containing four consecutive paired bases, followed by two mismatched bases and a unique 5′ sequence. The technique permits calculation of a ratio of telomere repeat copy number to a chosen internal control gene copy number as a relative measure of telomere length unit.

The SEQUENOM's EpiTYPER assay is a methylation quantification method which relies on MALDI–TOF MS.[17] The robustness of this approach for DNA methylation quantification has been previously confirmed by ulterior studies.[18–20] In the current report, this assay was used to quantify the methylation of *TP53*, *P21* and *P16* promoter regions.

The aim of this study was to investigate a potential link between promoter hypermethylation of the *TP53*, *P21* and *P16* genes and telomere length shortening. Paired breast tumor and adjacent normal breast tissues were analyzed by quantitative PCR and MALDI–TOF MS to measure relative telomere length and promoter methylation level of aforementioned genes, respectively. In addition, we compared telomere length in tumor tissues to traditional pathological parameters and clinical predictive markers.

Materials and methods

Samples

The study was approved by the local institutional review board. Cases with demonstrated *BRCA1* or *BRCA2* germline mutations or primary invasive breast carcinoma were excluded. Size of each tumor was evaluated by calculation of surface area (in cm^2) based on the measurement of its two greatest axes. Samples were obtained from 52 paraffin-embedded breast cancer tissues and 52 paired adjacent normal tissues. The corresponding embedded tissue in paraffin blocks of both tumor and adjacent normal tissue were sectioned in 5 μm thickness for immunohistochemical staining study. The pathological type, grading and staging were confirmed from the original microscopy slides, which were reviewed separately by two experienced pathologists. Staging and grading was evaluated according to the WHO histological classification.

Remaining part of the paraffin blocks were sectioned (thickness, 10–20 μm) from entirely neoplastic and adjacent normal tissue for DNA extraction.Three to five tissue sections (around 100 mg of tissue) were subjected for DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany).

Immunohistochemical Staining

The standard avidin–biotin–peroxidase-complex and heat-induced antigen retrieval using the microwave method was applied for all immunohistochemical staining. The analysis for the detection of estrogen receptor (ER), progesterone receptor (PR) and HER2/neu (C-ErbB-2) proteins was carried out on 5 μm-thick sections of paraffin-embedded tissue blocks using primary antibodies for ER (Dako Ltd, Cambridgeshire, UK; clone ID-5, 1:50 dilution), PR (Dako, clone PgR, 1:300 dilution) and HER2/neu antibody (Dako, clone PN2A, 1:200 dilution). Horseradish peroxidase–streptavidin was added and incubated for 30 min followed by dimethylaminoazobenzene (DAB) treatment for 10 min.

Appropriate positive and negative controls were included with each immunohistochemical staining. Normal breast tissues from the studied cases served as an additional internal control. The Quick Score method was used for semi-quantification of ER and PR status as follows: The slides were assessed for average degree of the staining at low power ($\times 10$), and the following scores were allocated: negative (0), weak (1), moderate (2) or strong (3). The percentage of cells with positive nuclei was counted at high power ($\times 40$), and the following scores were allocated: $<25\%$ (1), $25 \leq 50\%$ (2), $50 \leq 75\%$ (3) and $>75\%$ (4). HER2/neu immunoreactivity was scored according to the conventional scoring system as follows: (0) negative, (1+) negative, (2+) equivocal or weakly positive and (3+) strongly positive.

Quantitative Assessment of the Telomere Length

Telomere repeated sequences and glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) amplification was carried out for each sample by real-time PCR. *GADPH* was used as internal control (nuclear DNA (nDNA) reference) against which DNA quantification normalization was carried out. Primers were as follows: for *GAPDH,* forward 5′-CCCCACACAC ATGCACTTACC-3′ and reverse 5′-CCTAGTCCCA GGGCTTTGATT-3′; for telomere DNA (tDNA), forward 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTG GGTTTGGGTT-3′ and reverse 5′-GGCTTGCCTTAC CCTTACCCTTACCCTTACCCTTACCCT-3′.[16] Detection and quantification were carried out with the ABI Prism 7000 Sequence Detector and ABI Prism 7000 SDS software (Applied Biosystems, Foster City, CA, USA), respectively.

Reaction was carried out with 5 μl of template DNA, 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems) and 7.5 μl of each 10 μM primers, for a final reaction volume of 25 μl. Thermal cycling was carried out according to the following reaction

sequence: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 54 °C for 2 min for the telomere amplification, and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for *GAPDH*. Following amplification, a dissociation curve was drawn to confirm the specificity of the reaction. Standard and dissociation curves were generated with the ABI Prism 7000 SDS software. R^2 for each standard curve was >0.98.

We examined the amplification efficiency for both *GAPDH* and telomere DNA with a set of serial dilutions, specifying a tDNA and a 97-bp *GAPDH* amplicon concentrations ranging from 3.125×10^4 to 10 pg/μl (including 31 250, 6250, 1250, 250, 50 and 10 pg/μl). There was good correlation of tDNA and *GAPDH* signal on serial dilutions, with comparable efficiencies of amplification. The average threshold cycle number (Ct) values of *GAPDH* and tDNA were obtained from each case in this study. The relative telomere length was calculated using the average Ct of telomere DNA and *GAPDH* (ΔCt = Ct *GAPDH*−Ct tDNA) in the same sample as an exponent of 2 ($2^{\Delta Ct}$). All samples were run in parallel triplicate and the median value was used for calculations. In each run, a standard curve and a negative control (water) were included.

High-Throughput Methylation Analysis using Thymidine-Specific Cleavage Mass Array on MALDI–TOF Silico-Chip

The EpiTYPER assay using MALDI–TOF MS and MassCLEAVE reagent was assessed for high-throughput analysis of DNA methylation patterns of three tumor suppressor gene promoters (*TP53*, *P21* and *P16*) according to the previously published methods.[17,19–21]

Briefly, bisulfite conversion of the target sequence was obtained using the Epitect bisulfite kit (QIAGEN AG, Basel, Switzerland). Primers were designed to cover the promoter regions with the most CpG sites. A T7-promoter tag was added to the reverse primer and a 10mer-tag sequence was added to the forward primer to balance the PCR primer length.[20] Unincorporated dNTPs were dephosphorylated by alkaline phosphatase treatment (SAP; SEQUENOM, San Diego, CA, USA). Typically, 2 μl of the PCR were directly used as a template in a 5 μl of *in vitro* transcription reaction. In all, 20 U of T7 R&DNA polymerase (Epicentre, Madison, WI, USA) was used to incorporate dTTP in the transcripts. Ribonucleotides and dNTPS concentrations were 1 and 2.5 mmol/l, respectively. In the same step, the RNase A (SEQUENOM) was added to cleave the *in vitro* transcript (T-cleavage assay). The mixture was robotically dispensed (nanodispenser) onto silico chips preloaded with matrix (SpectroCHIP; SEQUENOM). Mass spectra were collected using a MassARRAY Compact MALDI–TOF (SEQUENOM) and spectra's methylation ratios were generated by the Epityper software v1.0 (SEQUENOM).

Statistical Analysis

Data analysis was carried out using the SPSS software (Statistical Software Package for Windows, version 17). The Shapiro–Wilk and Kolmogorov–Smirnov tests were used to data distribution analyzis. Both tests similarly demonstrated that our data set was not normally distributed ($P = 0.000$/0.000 by Shapiro–Wilk test and $P = 0.000$/0.001 by Kolmogorov–Smirnov test for tDNA assay and *GAPDH* assay, respectively). The relative telomere length is given as the median. Wilcoxon signed ranks test was used to compare the differences between ranks of each paired samples. The Mann–Whitney *U*-test and Kruskal–Wallis test were used to compare the shortened telomere length in the cancer tissues and paired normal tissues.

Quantitative methylation status of the three previously stated tumor suppressor promoters was compared with relative telomere length. Using the two-way hierarchical cluster analysis, the most variable CpG fragments for each gene were clustered based on pair-wise Euclidean distances and linkage algorithm for all studied samples according to the previously developed method.[19–21] For gene clustering, pair-wise similarity metrics were calculated for each gene separately based on methylation ratio of cancer tissues across the adjacent paired normal tissues. The procedure was carried out using the double dendrogram function of the Gene Expression Statistical System for Microarrays (GESS) version 7.1.19 (NCSS, Kaysville, UT, USA). The non-parametric Spearman's ρ-test was used for correlation study between telomere length and promoter hypermethylation of *TP53*, *P21* and *P16* gene promoters using SPSS software.

Results

Pathological Classification of Samples

According to pathological tumor type and immunohistochemical staining, we separated our patient' samples into two subgroups: ductal carcinoma and lobular carcinoma (Figure 1). Breast cancer characteristics, such as staging, histological grading, hormone receptor status and HER2/neu expression from breast cancer patients are listed in Tables 1 and 2.

Telomere Length Quantification and Methylation Analysis using Paraffin Embedded Tissues

The use of formalin-fixed, paraffin-embedded tissue sections, for which there is a significant degree of degradation, fragmentation and chemical modification of the nucleic acids, is a major limitation for this type of study[19,22] because it can reduce the

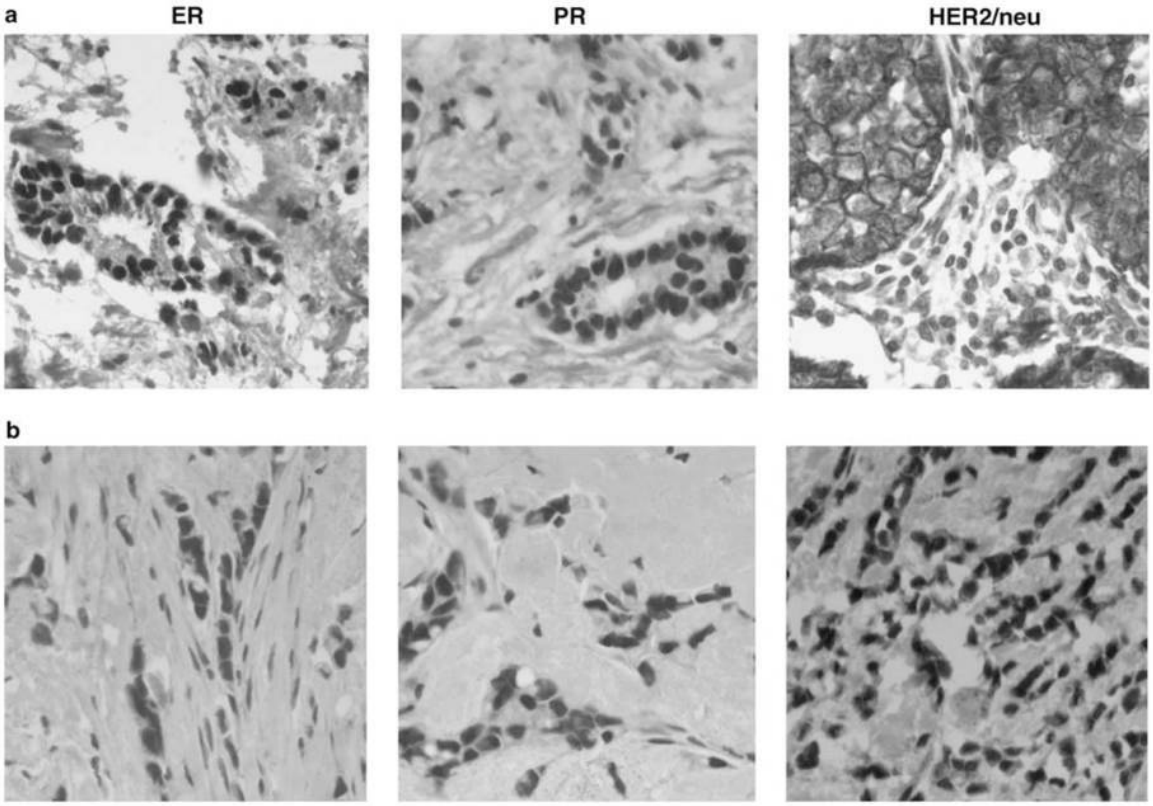

Figure 1 Immunohistochemical staining for estrogen receptor (ER), progesterone receptor (PR) and HER2/neu proteins. (**a**) Ductal carcinoma. (**b**) Lobular carcinoma.

sensitivity of the telomere length measurement.[23,24] In this study, we explored the feasibility of using paraffin-embedded tissues for the quantitative assessment of telomere length together with high-throughput methylation quantification on MALDI–TOF silico-chips.[20,21] Before carrying out telomere length analysis, yield of extracted DNA from formalin-fixed paraffin-embedded tissues was quantified using NanoDrop ND-1000 spectrophotometer (Biolab, Mulgrave, Vic, Australia). Median DNA quantities was determined as 37.92 ng/μl. Samples with <10 ng/μl of DNA were not considered for further experiments and DNA extraction for these samples was repeated.

Relative Telomere Length in Cancer and Paired Normal Breast Tissues

Data analysis demonstrates an 8.58 cycle difference ($\Delta\Delta$Ct, delta Ct) between normal and cancer tissues. The telomere length in cancer tissues was significantly lower than that in normal tissues (Mann–Whitney *U*-Test: $P<0.001$) (Figure 2a). Out of 52 paired samples, 41 pairs show telomere length in normal tissues significantly longer than telomere length in tumors (Wilcoxon signed ranks test: $P<0.001$).

Correlation Between Shortened Telomere Length and Other Prognostic Factors for Breast Cancer

In this study, associations between the ratio of telomere length in breast cancer tissues and traditional clinical parameters, such as age, tumor type, tumor size, lymph node involvement, extent of metastasis, stage, histological grading, receptor status and pathological biomarkers (HER-2/neu and PS2), were analyzed (Table 2). Shortened telomere length in breast cancer tissues was not associated with age (≥ 50 *vs* <50), tumor type (ductal *vs* lobular), tumor size (T1, T2 and T3), lymph node involvement (N0 and N$\geq$1), ER and PR status, HER-2/neu amplification nor presenilin 2 (PS-2) detection (Table 2). However, shorter telomere length was correlated with higher histological grading (grade I *vs* II and III, $P=0.007$) (Figure 2c) and distant metastasis ($P=0.035$) (Figure 3). We could not find any significant telomere length

Table 1 Clinical characteristics of patient's samples

Breast cancer tumor type	*Total no. of patients*	*Age (years) (mean ± s.d. (range))*	*Side of tumor*		*Tumor size (cm) (mean ± s.d. (range))*	*No. of patients with lymph node involvement*	*No. of patients with metastasis*	*Histological grade*		
			R	*L*				*Grade 1*	*Grade 2*	*Grade 3*
Ductal carcinoma	41	48 ± 11.2 (32–78)	19	22	3.6 ± 3.1 (0.8–12)	33	9	7	15	19
Lobular carcinoma	11	50 ± 10.9 (32–65)	6	5	3.25 ± 1.6 (1.5–6)	6	2	3	3	5

Table 2 Correlation between relative telomere length in cancer tissues and clinical parameters

Variables	*Group (cases)*	*Telomere length (median)*	P-*value*
Age (years)	<50 (33)	0.069	0.294[a]
	≥50 (19)	0.106	
Histological type	Ductal (41)	0.099	0.491[a]
	Lobular (10)	0.035	
Primary tumor	T1 (23)	0.069	0.397[b]
	T2 (16)	0.052	
	T3 (9)	8.439	
Lymph node involvement	Positive (39)	0.069	0.185[a]
	Negative (13)	0.271	
Distant metastasis	No metastasis (41)	4.291	0.035[a]
	Metastasis (11)	0.069	
Stage	I (6)	8.439	0.028[b]
	II (27)	0.541	
	III (8)	1.119	
	IV (11)	0.469	
Histological grading	G1 (10)	2.950	0.07[b]
	G2 (18)	0.036	
	G3 (24)	0.035	
ER	Positive (14)	0.271	0.342[a]
	Negative (18)	0.036	
PR	Positive (18)	0.332	0.138[a]
	Negative (14)	0.041	
HER2/neu	Positive (15)	0.069	0.664[a]
	Negative (17)	0.084	
P53	Positive (4)	2.587	0.185[a]
	Negative (29)	1.069	
PS-2	Positive (12)	0.114	0.836[a]
	Negative (20)	0.524	

ER, estrogen receptor; PR, progesterone receptor.
[a]Mann–Whitney *U*-test.
[b]Kruskal–Wallis test.

changes in the normal samples and their clinical prognostic parameters ($P>0.05$).

Methylation Status of Three Tumor Suppressor Genes Using MALDI–TOF MS

The quantitative methylation profile of three tumor suppressor promoters (*TP53*, *P21* and *P16*) was compared with relative telomere length in breast cancer and paired normal tissues. The CpG sites were analyzed by T-cleavage assay using MALDI–TOF MS. For these three studied promoters, we analyzed one amplicon per gene containing 83 CpG sites per sample (4316 CpG sites in total).[20] Using the two-way hierarchical cluster analysis, we demonstrated different levels of methylation in the three studied promoters in cancer and adjacent normal tissues (Figure 4). The results showed significant correlation between the telomere length shortening ratio in cancer tissues and the *TP53*, *P21* and *P16* promoters hypermethylation ($r=-0.33$, $P=0.001$; $r=-0.70$, $P<0.0001$ and $r=-0.71$, $P<0.0001$ respectively) (Figure 4).

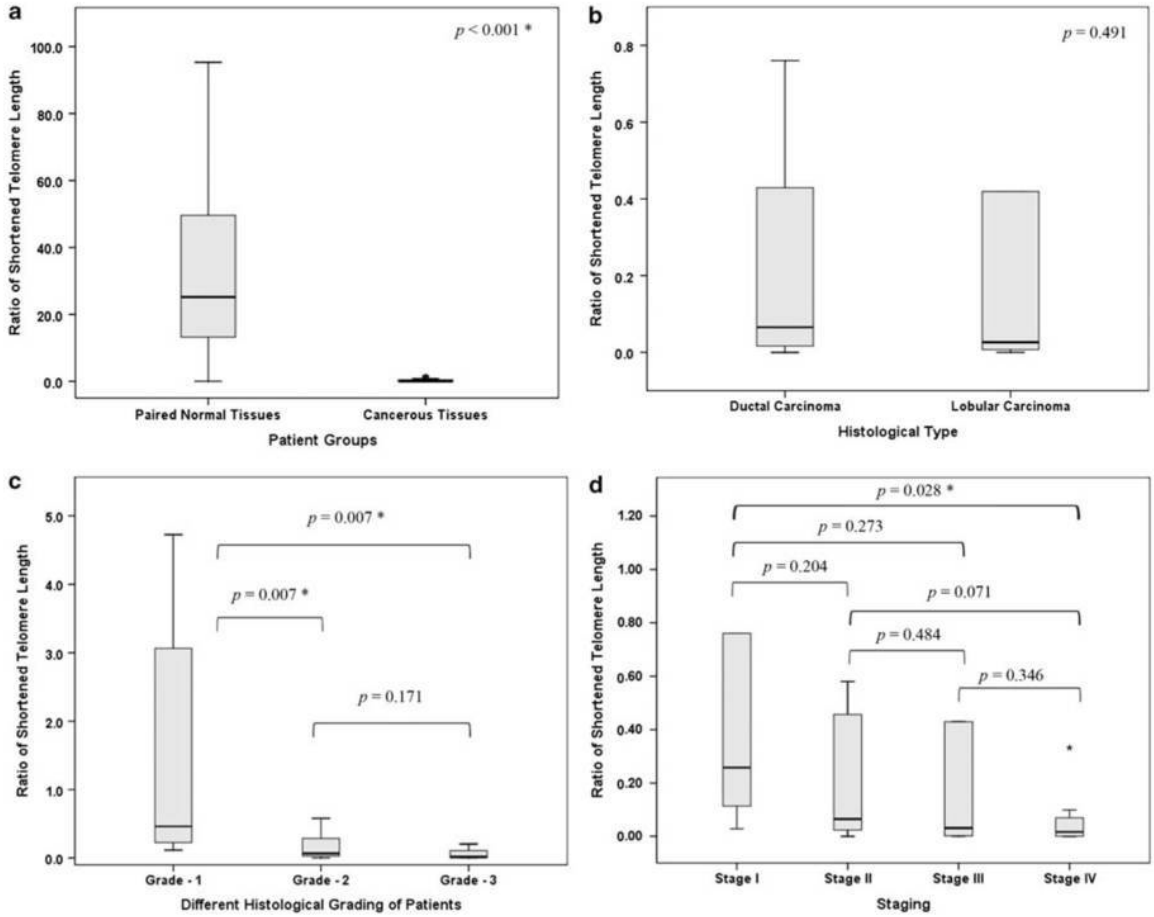

Figure 2 (**a**) Telomere length in the 52 breast cancer tissues and 52 paired normal tissues. (**b**) Telomere length in the two tumor types (ductal carcinoma and lobular carcinoma). (**c**) Telomere length relative to histological grade. (**d**) Telomere length relative to staging. (*significant correlation; Mann–Whitney *U*-test.)

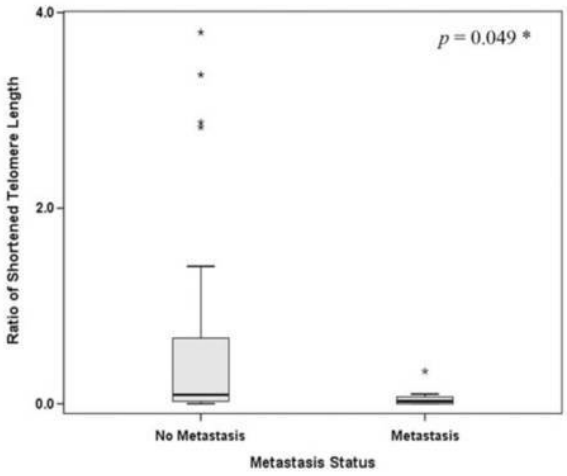

Figure 3 Telomere length in relation to metastasis status. (*Mann–Whitney *U*-test.)

Discussion

The association between telomere length alterations and cancer has been studied for decades. In the present study, telomere length and promoter methylation status of the p16/Rb and p53/p21 pathways were examined in different grades of breast cancer in 104 paired cancer and normal tissues from 52 patients with breast cancer. Our results show that the average telomere length in breast cancer tissue was significantly shorter than that in the adjacent normal tissue, especially in the histological grades II and III ($P<0.001$) (Figure 2c). Moreover, hypermethylation of *TP53*, *P21* and *P16* promoters significantly correlated with shortened telomere length in the cancer tissues ($P<0.001$) (Figure 4).

The most important function of telomeres is the maintenance of genomic integrity and stability.[1,25] Recently, Lin *et al*[24] reviewed the dynamics of telomere length in different types of cancers. Several studies examining telomere length in humans found

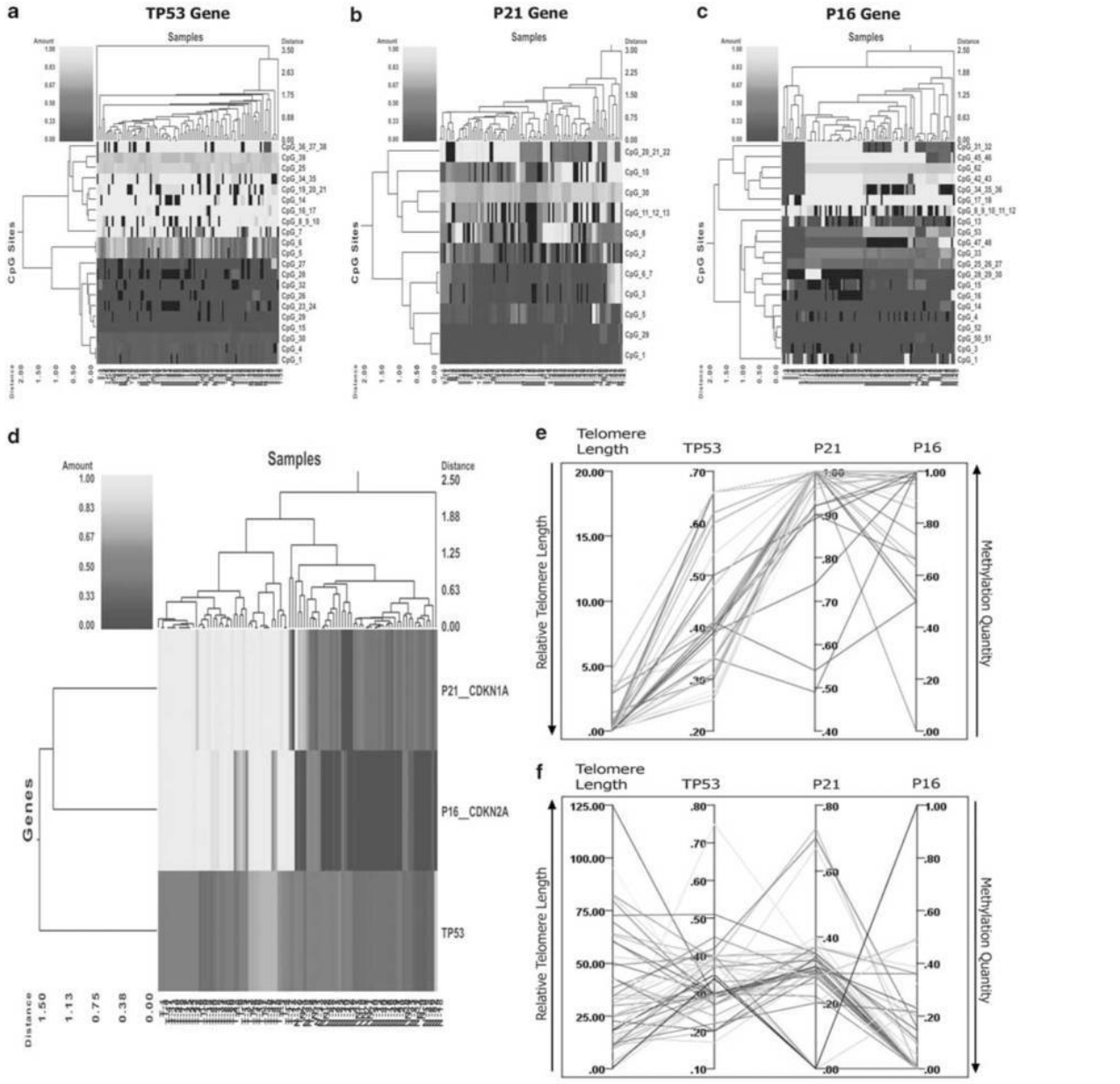

Figure 4 Correlation study between methylation profile of the three tumor suppressor genes (*TP53*, *P21* and *P16*) in cancerous tissues and paired normal tissues with relative telomere length. (**a–c**) High-throughput methylation analysis of three breast cancer-related genes by two-way hierarchical cluster analysis. Double dendrograms of the informative CpG sites for the paired samples of breast cancer patients. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0–100 and black clusters indicate CpG sites not analyzed; T: tumor tissue; N: normal tissue). (**d**) Double dendrogram presenting the methylation profiles of three studied genes (*TP53*, *P21* and *P16*). (**e**) Correlation study between relative telomere length with methylation profile of studied genes in the cancerous tissues. (**f**) Correlation study between relative telomere length with methylation profile of studied genes in the normal tissues. For color figure see online version.

that breast carcinomas had shorter telomeres than normal breast tissue, and high grade (grade III of III) invasive carcinomas had shorter telomeres than low grade (grade I) invasive carcinomas.[26] Our study on paired cancer and normal tissues concurs with those previous reports, and also demonstrates correlation between shortened telomere length and higher histological grading (Figure 2c and d).

Recently, Svenson *et al*[27] showed that telomere length in peripheral blood cells differs between

breast cancer patients and control subjects, and may serve as a significant prognostic biological marker. In general, malignant tumors show shorter telomeres than corresponding normal tissue, and telomere dysfunction has been indicated as a negative prognostic marker in solid tumors, including breast cancer.[28,29]

Telomere shortening in primary human cells leads to replicative senescence, which is regulated in part by effectors in the p16/Rb and/or p53/p21 pathways.[9,11,30] As well, p53/p21-mediated DNA damage signals are elicited by telomere dysfunction. However, there is still considerable debate on the exact role of telomere attrition on the p16 pathway during senescence.

Inhibition of the p16/Rb and/or p53/p21 pathways enables continuous cell division and critical telomere shortening, a phenomenon known as 'telomere crisis'.[15,31] Using the mass spectrometry on MALDI–TOF silico-chips, we determined quantitative methylation changes of *TP53*, *P21* and *P16* promoters in paired cancer and non-tumor samples. *P21* and *P16* promoters have been reported as being strongly hypermethylated in breast cancer tissues, compared with adjacent normal breast tissues.[20] This study shows significant correlation between telomere length shortening and *TP53, P21* and *P16* promoters methylation status (*TP53*:$r=-0.33$, $P=0.001$; *P21*:$r=-0.70$, $P<0.0001$ and *P16*:$r=-0.71$, $P<0.0001$) (Figure 4) in breast cancer tissues. Although our previous study indicated some hypermethylation level of the *TP53* promoter, this hypermethylation level was not significant in breast cancer tissues in comparison with adjacent normal tissues.[20] Our new finding shows a significant correlation between *TP53* hypermethylation and telomere shortening. This suggests that even low level of hypermethylation of *TP53* can influence telomere length shortening. Taken together, these findings could suggest that p16 and p53/p21 may function as a gatekeeper to prevent critical telomere shortening and genome instability.

Changes in telomere length in breast cancer may not be correlated with distant invasion.[32] However, our results showed a significant correlation between telomere length shortening and distant metastasis ($P<0.05$) (Figure 3). Although we could not find any significant correlation between telomere length and age (≥ 50 *vs* <50) in cancer tissue (Figure 5a), a significant shortened telomere length was observed in patients over the age of 50 years ($P<0.05$) (Figure 5b). This finding is in accordance with previous studies on telomere length and aging.[33,34]

It was reported that telomere length is reduced in histologically normal tissues distant at least 1 cm from the adjacent tumor margins.[35] Although the tissue appears normal, it harbors various genetic changes, including telomere dysfunction, that are reflective of the adjacent carcinogenesis process. This seriously complicates the use of histologically normal tissue as control.[35,36] Also examining whole tissue sections rather than examining telomere lengths by *in-situ* methodology, which permits distinction of lesional tissue from contaminating normal tissues is one of the limitations of used methodology.[37] To reduce the challenge of contaminating normal tissues, the paired normal samples in our study were selected with distances more than 1.5 cm to the adjacent tumor tissues. In one report, moderate telomere shortening was observed in approximately 50% of histologically benign breast duct lobular units.[37] This kind of telomere shortening might be the result of physiological proliferation, and may delineate the cells at risk for subsequent malignant transformation, but in our work we could not find significant telomere shortening in the far adjacent normal tissues.

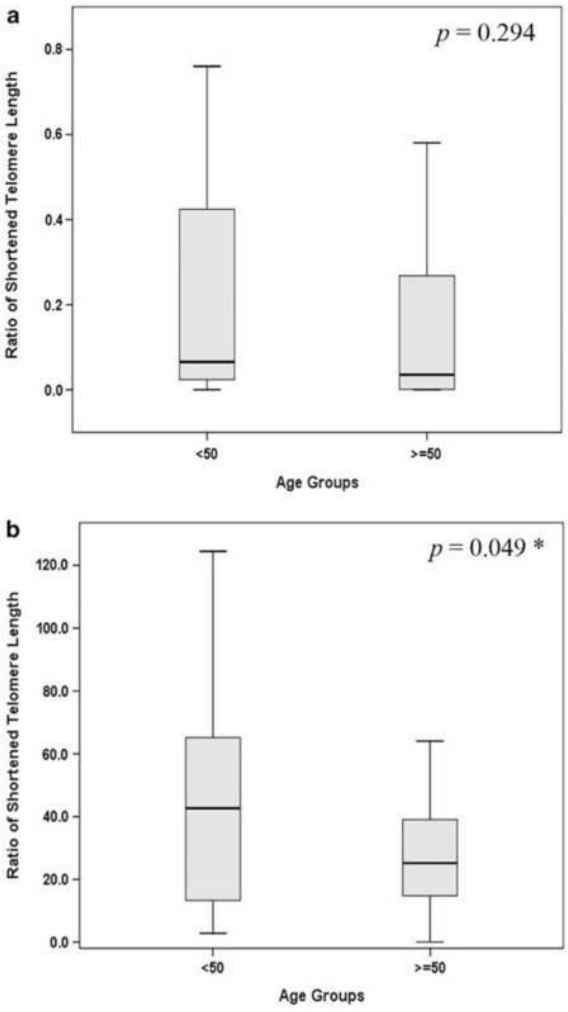

Figure 5 Comparison of telomere shortening in patient's samples separated according to age, in (**a**) cancer tissues (**b**) paired normal tissues. (*significant correlation; Mann–Whitney *U*-test.)

In conclusion, our data suggests that shortened telomere length is significantly correlated with carcinogenesis. Moreover, telomere length is also significantly shorter in cancer tissues from patients with distant metastasis compared with those from patients without metastasis. Promoter hypermethylation of the p16/Rb and p53/p21 pathways showed significant correlation with telomere shortening. The involvement of these cell-cycle regulator pathways may allow for continuous cell division and critical telomere shortening. Shortened telomere length and hypermethylation of p53, p21 and p16 promoters might serve as biomarkers in breast cancer.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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3.3 Published research article:

Methylation profile of TP53 regulatory pathway and mtDNA alterations in breast cancer patients lacking TP53 mutations

Authors: Zeinab Barekati, Ramin Radpour, Corina Kohler, Bei Zhang, Paolo Toniolo, Per Lenner, Qing Lv, Hong Zheng and Xiao Yan Zhong

Journal: Human Molecular Genetics. 2010, 1; 19(15): 2936-46.

Summary: The present study investigated promoter hypermethylation of *TP53* regulatory pathways, *P14^{ARF}/MDM2/TP53*/*PTEN*, providing a potential link between epigenetic changes and mitochondrial DNA alterations in breast cancer patients lacking a *TP53* mutation. The possibility of using the cancer-specific alterations in serum samples as a blood-based test was also explored. Our finding showed association between two hypermethylated genes, *P14^{ARF}* and *PTEN,* and mtDNA depletion as well as high frequency of somatic mutation in the tumour tissues compared to the matched normal tissues. Taken together these data provides an insight into understanding of p53 dysfunction based on the epigenetic silencing of the crucial genes involved in autoregulatory loops. Additionally, release of significant aberrant methylated *PTEN* in matched serum samples could suggest a promising biomarker for breast cancer.

Author contributions: Zeinab Barekati was involved in experimental design, performing the study, data analysis, interoperating data and writing the manuscript.

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Methylation profile of *TP53* regulatory pathway and mtDNA alterations in breast cancer patients lacking *TP53* mutations

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The present study investigated promoter hypermethylation of *TP53* regulatory pathways providing a potential link between epigenetic changes and mitochondrial DNA (mtDNA) alterations in breast cancer patients lacking a *TP53* mutation. The possibility of using the cancer-specific alterations in serum samples as a blood-based test was also explored. Triple-matched samples (cancerous tissues, matched adjacent normal tissues and serum samples) from breast cancer patients were screened for *TP53* mutations, and the promoter methylation profile of $P14^{ARF}$, *MDM2*, *TP53* and *PTEN* genes was analyzed as well as mtDNA alterations, including D-loop mutations and mtDNA content. In the studied cohort, no mutation was found in *TP53* (DNA-binding domain). Comparison of $P14^{ARF}$ and *PTEN* methylation patterns showed significant hypermethylation levels in tumor tissues ($P < 0.05$ and <0.01, respectively) whereas the *TP53* tumor suppressor gene was not hypermethylated ($P < 0.511$). The proportion of *PTEN* methylation was significantly higher in serum than in the normal tissues and it has a significant correlation to tumor tissues ($P < 0.05$). mtDNA analysis revealed 36.36% somatic and 90.91% germline mutations in the D-loop region and also significant mtDNA depletion in tumor tissues ($P < 0.01$). In addition, the mtDNA content in matched serum was significantly lower than in the normal tissues ($P < 0.05$). These data can provide an insight into the management of a therapeutic approach based on the reversal of epigenetic silencing of the crucial genes involved in regulatory pathways of the tumor suppressor *TP53*. Additionally, release of significant aberrant methylated *PTEN* in matched serum samples might represent a promising biomarker for breast cancer.

INTRODUCTION

Breast cancer is the most common malignancy among females worldwide. A large number of reports have discussed the important role of *TP53* alterations in breast cancer. The *TP53* tumor suppressor gene has a central role in cell cycle regulation (1), DNA repair (2,3), senescence and apoptosis (4), differentiation and development, and prevention of cancer (5).

Recent studies showed that the *TP53* gene contributes to transcription or replication regulatory mechanisms of mitochondria and also boosts mitochondrial genomic stability via stimulation of base excision repair (6–8). Human mitochondrial DNA

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(mtDNA) mutation can be induced by generation of reactive oxygen species (ROS). An increase of mtDNA sensitivity to DNA-damaging oxidative stress such as ROS has been shown to result via deregulation of p53 protein in cancer cells (8,9). Most of the mtDNA mutations occur in the D-loop region, the major replication and transcription site of both the heavy and light strands of mtDNA (10–12). Previous studies in this area showed that somatic mutation in the DNA-binding domain of the *TP53* gene contributes to mtDNA mutation and mtDNA depletion that indicates a possible role in tumorigenesis of breast cancer patients (8,13,14).

Deregulation of the tumor suppressor *TP53* gene during pathogenesis of most human cancers has been reported via mutation or through alteration in encoding regulators of *TP53* (15–17). The frequency of *TP53* mutations detected in breast cancer is ~20% that were mostly determined by sequence analysis of the DNA-binding domain (exons 5–8) (18,19). This frequency is lower than that of many other cancers. Other than *TP53* mutation, alteration of upstream and/or downstream *TP53* regulators could be another plausible mechanism for inactivation and suppression of this gene in breast cancer. Recently, association of promoter hypermethylation with mtDNA alteration and nuclear DNA crosstalk has been reported in cancer (20). Taken together, this can provide new insights into epigenetic silencing of some of the regulatory pathways, leading to the deregulation of p53 protein which most probably influences the mtDNA vulnerability in breast cancers lacking *TP53* mutation. The *P14^{ARF}/MDM2/TP53* and *PTEN* pathways are the two critical *TP53* control pathways, and alteration of either pathway can induce a variety of cancers and they are thus the focus of many breast cancer investigations (5,21–25).

Regarding the *P14^{ARF}/MDM2/TP53* pathway, Mdm2 normally inactivates the tumor suppressor *TP53* and, in response to stress, regulates the duration and activity of p53 protein. Mdm2 protein binds directly to *TP53* to inhibit transcription and export p53 from the nucleolus to the cytoplasm, resulting in the degradation of p53 by ubiquitination (26,27). The role of p14^{ARF} in the *TP53* pathway is to indirectly regulate the level of p53 protein. p14^{ARF} protein inhibits degradation of p53 protein by keeping Mdm2 in the nucleolus and also restricting E3 ubiquitin protein (26).

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is an essential component of the *TP53* gene response upon DNA damage. Pten protein indirectly regulates p53 function through keeping Akt inactive and making Mdm2 incapable of translocating into the cell nucleus for degradation of p53 (28,29). Indeed, Pten protects p53 even in the presence of Mdm2 by increasing p53-mediated transcription (28) as well as binding to p53, leading to an increase in the half-life of p53 (30).

The aim of the present study was to investigate the promoter methylation profile of the regulatory pathways of *TP53* (*P14^{ARF}/MDM2/TP53*, as well as *PTEN*), providing the potential link between epigenetic changes and mtDNA alteration in tumorigenesis in breast cancer patients lacking *TP53* mutation on the DNA-binding domain. The pathway analysis was performed to verify which of the selected genes were most relevant in breast cancer. Triple-matched samples from breast cancer patients (cancerous tissues, matched normal tissues and serum samples) were subjected to mutation analysis of the DNA-binding domain of *TP53*, assessment of the promoter methylation profile of four candidate genes (*P14^{ARF}/MDM2/TP53/PTEN*) and mtDNA alteration, including D-loop region integrity and mtDNA content to measure the correlation of the aforementioned subjects in the studied cohort. This approach presented the cancer-specific alterations in tissues followed by exploration of the possibility of using the alterations in corresponding serum samples as a blood-based test.

RESULTS

P14^{ARF}/MDM2/TP53 and *PTEN* pathway analysis

First, the network of the candidate genes was analyzed as a function of cellular interactions and breast cancer development. The direct interaction pathway analysis displayed specific upstream and downstream of four candidate genes that identify common targets and regulators for these genes. Additionally, it showed the binding partner of these genes and behaving role in breast cancer (Fig. 1). Signaling analysis including hierarchical and direct force analysis are shown in Supplementary Material 1. This analysis provided an approach to compare different kinds of interactions, including protein–protein interactions as well as genetic interaction.

TP53 mutation screening

The analysis of the results after direct sequencing of exons 5–8 of the *TP53* gene revealed no mutation in the 44 normal and cancerous breast tissue samples from 22 patients compared with the *TP53* GenBank sequence (NC_000017 [gi:224589808]).

Quantitative methylation profiles of *P14^{ARF}/MDM2/TP53* and the *PTEN* promoter using MALDI-TOF MS

In this study, we analyzed the methylation patterns of four breast cancer candidate genes in 66 triple-matched samples (cancerous tissues, matched normal tissues and serum samples) from 22 breast cancer patients. For all the genes, 1 amplicon per gene and 117 CpG sites in total per sample (total of 7020 sites in 66 analyzed samples) were analyzed (Table 1; Supplementary Material 3). In the present study, a low degree of methylation with mean methylation levels <30% was considered as hypomethylation and a high degree of methylation with mean methylation levels >70% was considered as hypermethylation.

Using two-way hierarchical cluster analysis, we found different levels of methylation between tumor tissue, the matched serum and the normal tissue samples. The methylation quantification data for each studied gene are illustrated in Supplementary Material 3. The methylation alteration level of the individual CpG sites or clusters per gene showed a variable signature in the studied cases (Supplementary Material 3); the mean methylation quantity of the informative CpG sites that represent valuable differences in each case was used to figure out the promoter alterations in the candidate genes. Hierarchical cluster analysis profiling of the promoter alterations of the four studied genes is shown in Figure 2A.

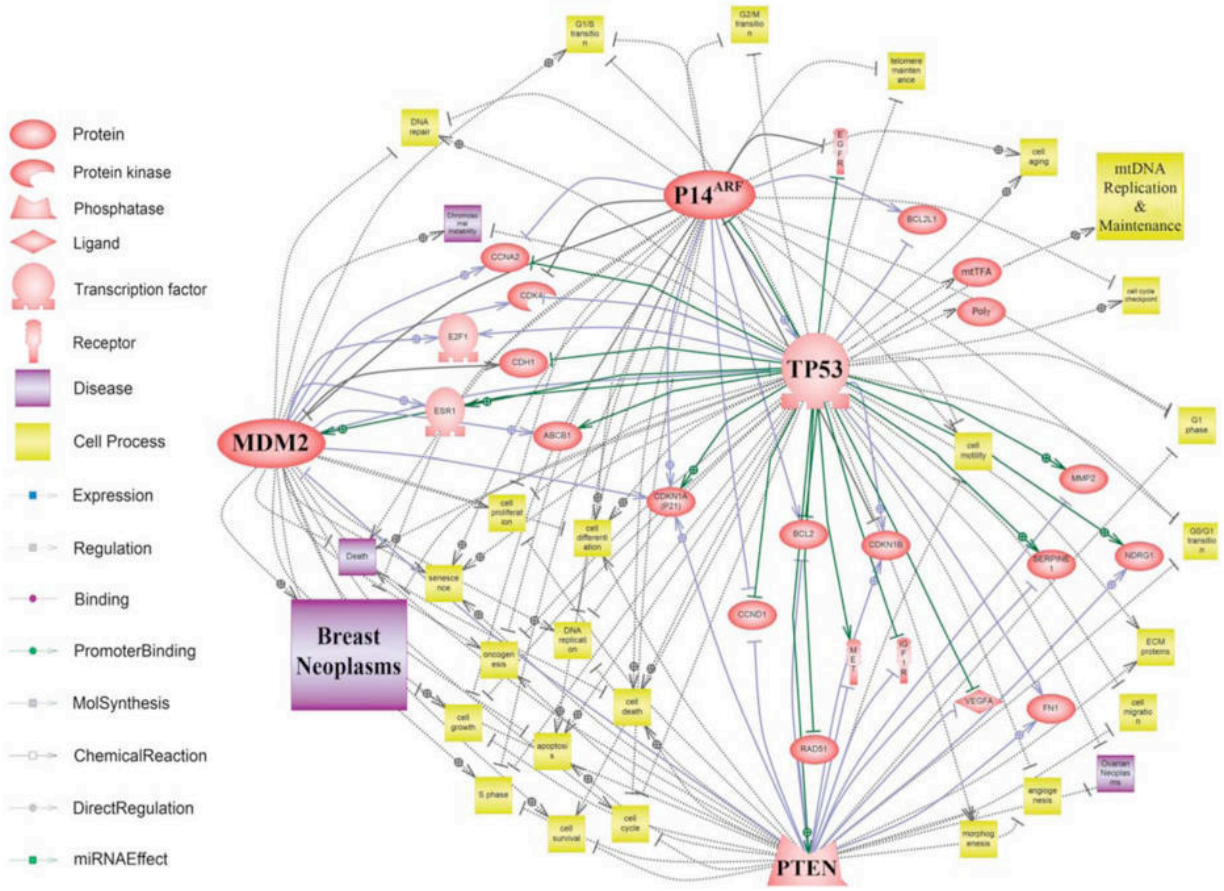

Figure 1. Direct interaction pathway analysis of four candidate genes related to breast cancer.

Table 1. High-throughput methylation analysis of CpG sites in the amplicons of the studied genes in breast cancer

Gene	Amplicon size (bp)	Total no. of CpG sites in amplicon	No. of analyzed CpG sites in amplicon	No. of analyzed CpG sites per amplicon	
				Single sites	Composite sites
MDM2	303	39	27	7	20
P14^{ARF}	425	36	26	6	20
PTEN	451	34	29	10	19
TP53	449	39	35	17	18

The *in silico* digestion was performed for the T-cleavage assay. The percentage of total CpG sites in the amplicon is divided into single sites (single CpG sites) and composite sites (two or more adjacent CpG sites fall within one fragment, or when fragment masses are overlapping).

The *MDM2* oncogene for both the tumor and normal tissues showed a relatively comparable hypomethylation pattern whereas matched serum was significantly different compared with normal tissue ($P < 0.05$). Evaluation of the methylation proportion of *P14^{ARF}* presented significant hypermethylation in tumor tissue versus normal tissue and in matched serum versus tumor tissue ($P < 0.05$ and <0.01, respectively). Comparison of the *PTEN* methylation pattern showed significantly higher hypermethylation levels in tumor tissue and matched serum than the normal tissue ($P < 0.01$ and <0.05, respectively). Our data showed significant concordance between the tumor and serum *PTEN* methylation profile (Spearman's rho test; $P < 0.05$). Methylation analysis of *TP53* showed no significant differences between tumor tissue and matched serum versus normal tissue whereas matched serum and tumor tissue revealed significant difference ($P = 0.01$; Fig. 2B).

Schematic comparison of methylation rate of analyzed genes with upstream regulatory sequence

The methylation rate and localization of each CpG site were compared with upstream sites for regulatory enhancers, CAAT box, GC box, transcription factor IIB recognition elements, TATA box, initiation site of transcription and downstream promoter elements. Figure 2C schematically compares the upstream regulatory sequences (in the range of -1000 to $+100$) with the methylation value of informative CpG sites of

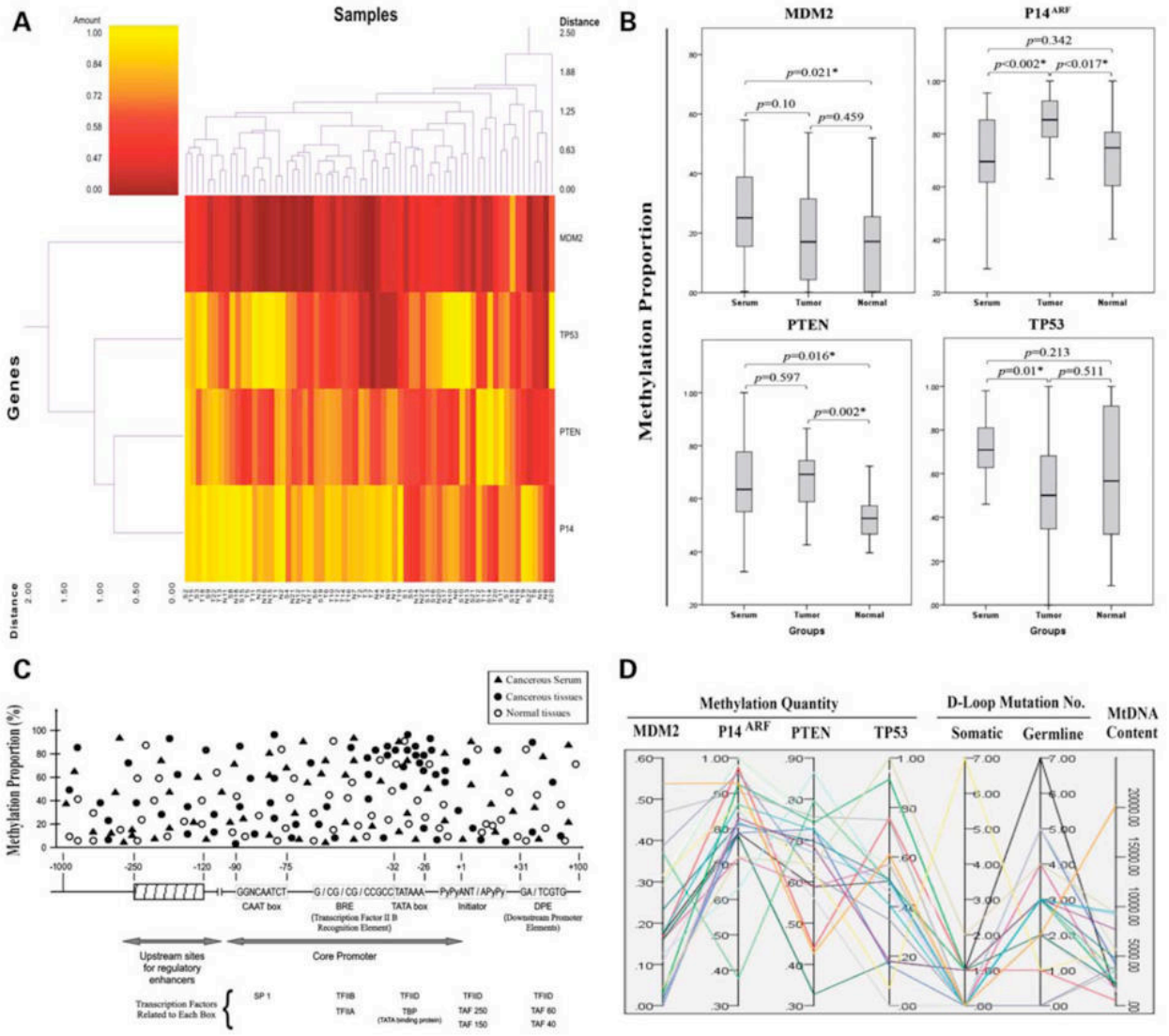

Figure 2. (**A**) Double dendrogram presents the methylation profiles of the four studied genes in triple-matched samples from 22 breast cancer patients (red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0 to 100). (**B**) Comparison between quantitative analysis of methylation for the studied genes in triple-matched samples (*significant correlation; Mann–Whitney *U*-test). (**C**) Comparison of the methylation ratio and approximate position of informative CpG sites of the four studied genes according to the recognition sites of the transcription factors (in the range of −1000 to +100) in the triple-matched samples. (Each dot in the map corresponds to the mean amount of CpG site methylation in all cases of each studied group.) (**D**) Correlation study between methylation quantity, D-loop mutations and mtDNA content.

the four studied genes together. Accumulation of some hypermethylated CpG sites around the TATA box region was observed for tumor-derived samples. In serum and normal samples, the CpG sites were differentially methylated and located randomly in the 5′-UTRs of the studied genes (Fig. 2C).

D-loop region mutation screening

The results of the D-loop region after direct sequencing in a pair study of normal tissue versus tumor were analyzed and compared with the human mitochondrial sequence (GenBank sequence NC_012920 [gi:251831106]). D-loop region mutation screening showed somatic mutations in 36.36% (8 of 22) of studied cancerous samples. Six of the eight cases showed a single somatic mutation, whereas of the remainder one had 2 and the other had 7 somatic mutations. The prevalence of detected germline mutations in the D-loop region was markedly higher at 90.91% (20 of 22) than somatic mutations and with mostly multiple germline mutations in each case. A total of 82 mutations were detected in the D-loop region of the studied samples, including 15

Table 2. Summary of the D-loop mutations found in breast cancer patients

D-loop alteration	Mutation name	Mutation frequency (%)
Somatic	64 C > C/T (H)	1 (4.54)
	146 T > C	1 (4.54)
	152 T > C	1 (4.54)
	199 T > C	1 (4.54)
	235 A > G	1 (4.54)
	256 C > G	1 (4.54)
	257 A > G	1 (4.54)
	16290 C > T	1 (4.54)
	16291 C > T	1 (4.54)
	16311 T > C	2 (9.09)
	16319 G > A	2 (9.09)
	16389 G > G/C (H)	1 (4.54)
	16519 T > C	1 (4.54)
Germline	41 C > C/T (H)	1 (4.54)
	83 A > A/C (H)	1 (4.54)
	84 A > A/C (H)	1 (4.54)
	146 T > C	3 (13.64)
	150 C > T	3 (13.64)
	152 T > C	1 (4.54)
	199 T > C	1 (4.54)
	204 T > C	2 (9.09)
	207 G > A	3 (13.64)
	210 A > G	1 (4.54)
	235 A > G	2 (9.09)
	248 Del A	1 (4.54)
	297 A > C/A (H)	2 (9.09)
	390 A > G	1 (4.54)
	398 C > T	1 (4.54)
	489 T > C	4 (18.18)
	16278 C > T	2 (9.09)
	16290 C > T	3 (13.64)
	16291 C > T	1 (4.54)
	16304 T > C	2 (9.09)
	16311 T > C	4 (18.18)
	16317 A > G	1 (4.54)
	16319 G > A	3 (13.64)
	16357 T > C	1 (4.54)
	16362 T > C	11 (50)
	16438 G > A	1 (4.54)
	16497 A > G	1 (4.54)
	16519 T > C	8 (36.36)

H, heterozygosity; Del, nucleotide deletion.

somatic mutations and 67 germline mutations. The details of the prevalence of germline and somatic mutations are provided in Table 2, and the spectrum of the mutation along the length of the D-loop is illustrated in Figure 3.

Mitochondrial content in triple-matched samples

The mtDNA contents of the studied, cancerous and normal breast tissue, as well as serum samples were measured using multiplex quantitative real-time PCR. The mtDNA content results obtained from cancer tissues were significantly lower than that of the normal tissues, with 11.88-fold changes ($P < 0.01$). Nineteen of 22 cancer tissues compared with normal tissues had lower mtDNA content (86.36%). The content of mtDNA in matched serum was lower than in normal and cancer tissues. The mtDNA content of matched serum was significantly lower than that of normal tissues (Spearman's rho test; $P<0.05$; Fig. 4).

Relationship of *TP53*/*P14*/*MDM2* and *PTEN* methylation profile, mtDNA alteration and clinicopathological parameters

The correlation of the methylation proportion of the studied genes to mtDNA alteration (D-loop mutations and mtDNA content) is summarized in Figure 2D. A negative correlation between germline mutation number and *MDM2*, as well as $P14^{ARF}$ methylation profile in tumor tissues has been found (Spearman's rho test; $P < 0.05$).

The mtDNA content showed no difference between patients with and without D-loop region mutations, suggesting that the mtDNA depletion may not be a result of the mutations (Fig. 4).

Clinicopathological parameters with our findings were analyzed (Supplementary Material 4). Breast cancer patients with the ILC histological type showed significant association with somatic mutations of the D-loop region ($P < 0.05$). In matched serum samples, patients with histological grade II had a higher proportion of hypermethylation of the *PTEN* gene and lower mtDNA content than histological grade III ($P < 0.05$). Serum samples of patients with C-ERB2 expression showed a greater proportion of hypermentylation of $P14^{ARF}$ than patients lacking C-ERB2 expression ($P < 0.05$).

DISCUSSION

p53 dysfunction can be compromised by *TP53* mutation, methylation or gene alteration of crucial p53 regulators. The majority of *TP53* mutation occur in the DNA-binding domain (exons 5–8); this domain has a critical role for the biological activity of p53 (31). The *TP53* mutation occurs in approximately 20% of breast cancer cases (19), and this frequency is lower than that of the other types of cancer. In the present study, the results showed no mutation in the *TP53* gene (DNA-binding domain) of the studied cohort, thus these samples were subjected to further epigenetic analysis.

Apart from *TP53* mutation, alteration of upstream and/or downstream regulatory factors could be another plausible mechanism for *TP53* inactivation and suppression in breast cancer. To find the molecular mechanism involved in tumor suppressor p53 protein deregulation of breast cancer patients lacking *TP53* mutation, we assessed epigenetic alteration of $P14^{ARF}$/*MDM2*/*TP53* and *PTEN*, important regulatory pathways of p53. Aberrant methylation of CpG islands is the most common alteration in human cancer (33). In the present study, the promoter methylation profile of four candidate genes, $P14^{ARF}$, *MDM2*, *TP53* and *PTEN*, was analyzed in 66 samples (breast tumors, matched normal tissue and serum samples). In tumor tissue, the *MDM2* oncogene showed hypermethylation patterns comparable with normal tissues. Comparison of $P14^{ARF}$ and *PTEN* methylation patterns showed significant hypermethylation levels in tumor tissues when compared with the normal tissues ($P < 0.05$ and <0.01, respectively) while the *TP53* tumor suppressor gene was not hypermethylated. Additionally, significant accumulation of hypermethylated CpG sites in the promoter region was observed for tumor tissues, which might suggest prevention of RNA polymerase action at transcriptional promoter

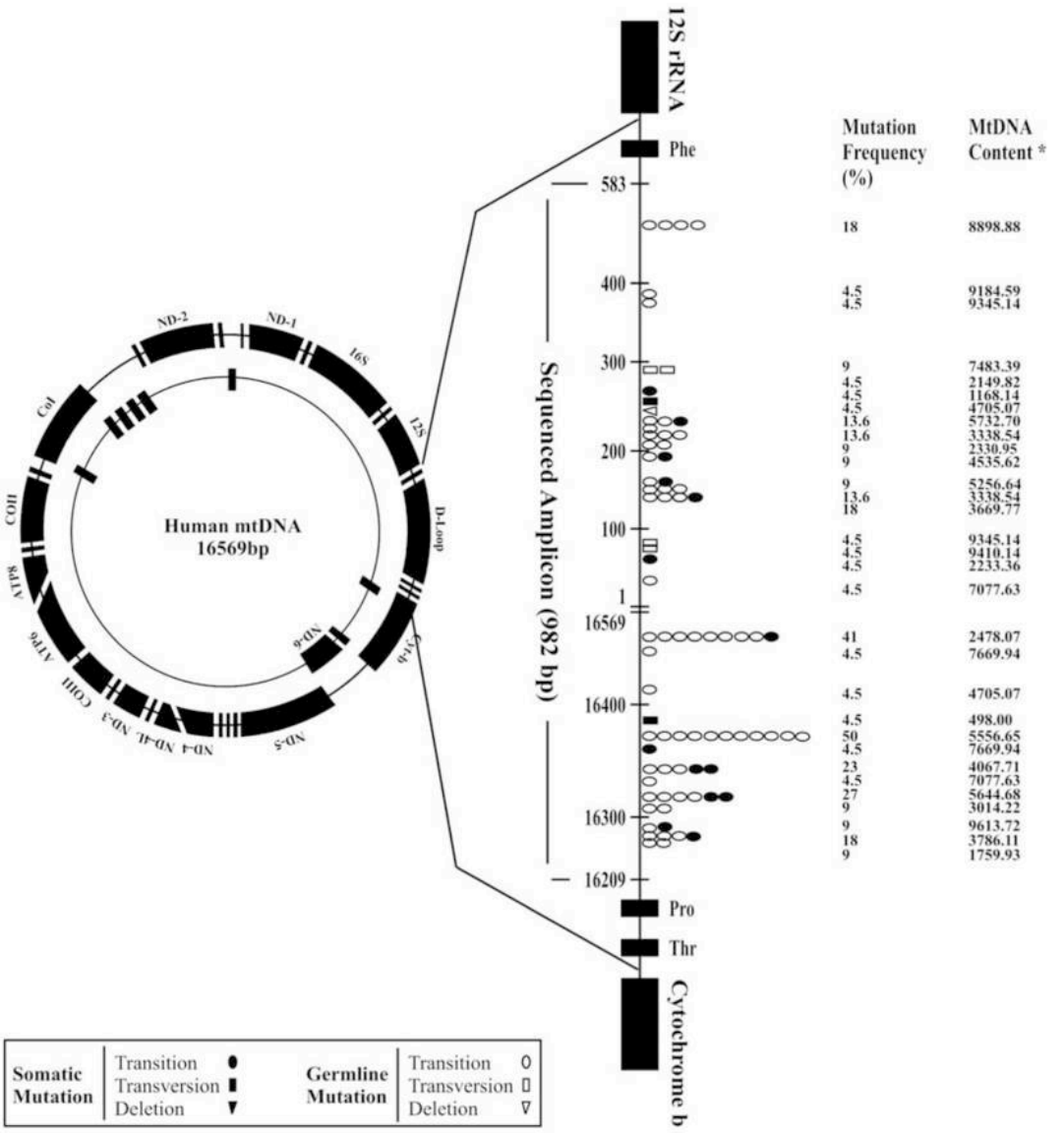

Figure 3. Distribution of the mutations in the D-loop region and mtDNA content. The asterisk indicates the median of mtDNA content of the patients who have the mutation in the same corresponding locus.

regions of *P14*^{ARF} and *PTEN* tumor suppressor genes leading to gene silencing. These results are in line with recent reports based upon methylation status and aberrant expression. Previous studies have investigated frequent aberrant methylation patterns of *P14*^{ARF} and a down-regulation of this tumor suppressor gene in breast tumors (21,34). Hypermethylation of the promoter of *PTEN* has also been found in breast carcinoma that led to *PTEN* inactivation in a subset of breast cancers (35–37).

Pathway analysis served as the important step toward an improved understanding of the relationship of studied genes in breast cancer. This investigation suggests the possibility of p53 deregulation probably by loss of *P14*^{ARF} and *PTEN* or by overexpression of *MDM2*. Mdm2 is responsible for inactivation of tumor suppressor *TP53* by inhibition of transcription and export of p53 from the nucleolus to the cytoplasm, resulting in the degradation of p53 by ubiquitination (26,27). Overexpression of the *MDM2* gene can result in an excessive inactivation of p53 and has been reported in 73% of breast carcinomas (22,23). p14^{ARF} protein, by keeping Mdm2 in the nucleolus and also restricting E3 ubiquitin protein, inhibits degradation of p53 (26). Indirect activation of p53 by p14^{ARF} inhibits growth of abnormal cells and prevents cancer (38). Indeed, p53 by binding to the *PTEN* promoter

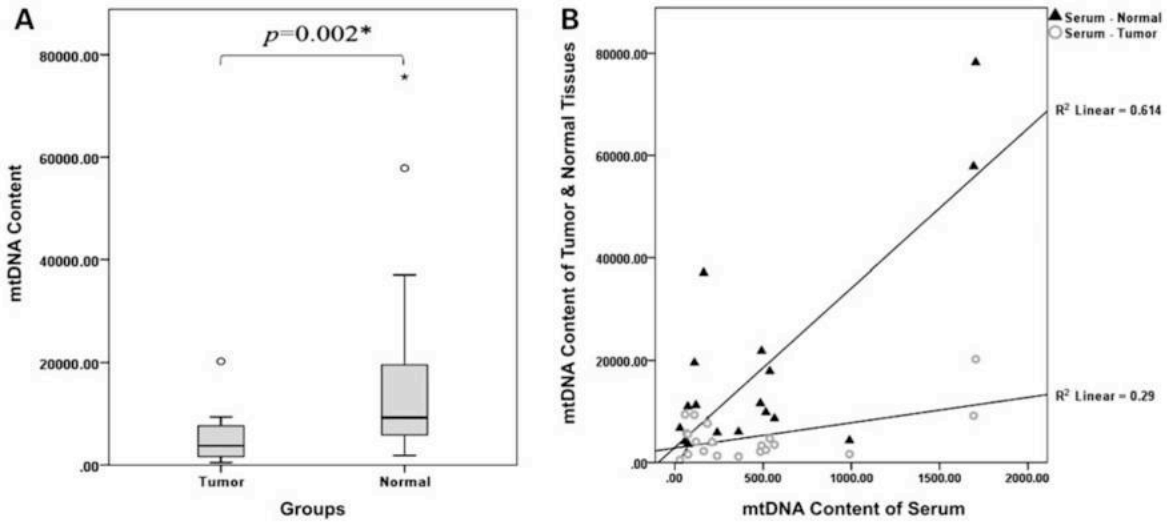

Figure 4. MtDNA content. (**A**) Comparison between mtDNA content in tumor and adjacent normal tissues. (**B**) Correlation of mtDNA content in serum versus tumor and normal samples. (* significant correlation; Mann–Whitney *U*-test).

leads to a positive feedback loop that decreases AKT activity by Pten and as a result inactivates Mdm2 and protects p53 from deregulation (39). Furthermore, Pten protein protects p53, even in the presence of Mdm2, by increasing *TP53*-mediated transcription (28) and also by binding to p53, leading to an increased p53 half-life (30). These findings emphasize the importance of an epigenetic alteration-mediated silencing of the tumor suppressor candidate genes and suggest that alteration of these genes could be a cause of p53 deregulation. These pathways might be targets for therapeutic strategies based on reversal of epigenetic silencing in breast cancer.

To determine the mechanistic link between inactivated tumor suppressor p53 through epigenetic silencing regulatory pathways and mtDNA alteration in cancer kinetics, we assessed mtDNA damage including D-loop region integrity and mtDNA content in breast cancer patients lacking *TP53* mutation. mtDNA has a higher mutation rate than nuclear DNA, due to its closeness to the respiratory chain, lack of histone protein and imitated DNA repair capacity. Most of the mutations occur in the D-loop, a major control site of replication and transcription for both the heavy and light strands of mtDNA (10–12). Association of *TP53* alteration with mtDNA damage has been reported in a breast cancer patient (13). Our data present a high rate of D-loop region mutations (36.36% somatic and 90.91% germline mutations) in breast cancer patients.

Depletion of mtDNA content has been investigated in breast cancer and it has been attempted to correlate this to mtDNA somatic mutation (18,40–43). The present data showed a significant mtDNA depletion in tumor tissues compared with normal tissues ($P < 0.01$). It has been demonstrated that the depletion of mtDNA resulted from a loss of p53 function (8). The mtDNA damage in the studied cohort, D-loop mutations and mtDNA depletion, might be explained by the indirect effect of p53 inactivation on mtDNA vulnerability. Additionally, we found an inverse correlation between germline mutations and *MDM2* and $P14^{ARF}$ hypermethylation in tumor tissues which might suggest a susceptibility to p53 inactivation in breast cancer patients with a background of D-loop mutations. Furthermore, breast cancer patients with the ILC histological type showed a significant association with somatic mutations of the D-loop region ($P < 0.05$).

Comparison of clinicopathological parameters with tumor-specific methylation changes and mtDNA content did not reveal additional information, and this remains to be determined in a larger cohort.

In the present study, in order to find cancer-specific markers in tissues for the development of a blood-based test, we assessed epigenetic alterations and mtDNA changes in matched serum samples of the studied cohort. Epigenetic alterations in serum of different cancers have been reported (44–49). DNA from solid tumors are released into the systemic circulation most probably through cellular necrosis or apoptosis (50). The present study investigated the proportion of methylation of four candidate genes in serum samples. The $P14^{ARF}$ tumor suppressor genes showed higher hypermethylation levels than the normal tissues, though this failed to reach the statistical significance. The hypermethylation level of $P14^{ARF}$ in serum samples was lower than in tumor tissues; this trend has also been reported previously (34). The proportion of *PTEN* methylation was significantly higher in matched serum than in the normal tissues, with a significant concordance between the tumor and serum *PTEN* methylation profile ($P < 0.05$), suggesting a possible use of the marker for the development of a blood-based test with the goal of patient management. Assessment of mtDNA content revealed depletion of mtDNA in matched serum versus control tissues ($P < 0.05$). Using mtDNA depletion as a blood-based test, a biomarker for breast cancer patients was suggested in a previous publication (42). Release of significant aberrant methylated *PTEN* in blood might represent

a promising biomarker for breast cancer with ease of access to methylation analysis.

In conclusion, the obtained results from breast cancer patients suggest hypermethylation of *P14^{ARF}* and *PTEN* could break down the *P14^{ARF}*/*MDM2*/*TP53* and *PTEN* regulatory pathways, resulting in p53 inactivation in breast cancer patients lacking *TP53* mutation in the DNA-binding domain. Moreover, mtDNA damage can be affected by p53 deregulated through epigenetic silencing of the studied pathways. These data can provide an insight into the management of a therapeutic approach based on reversal of epigenetic silencing of the crucial genes involved in regulatory pathways of the *TP53* tumor suppressor gene. Additionally, release of significant aberrant methylated *PTEN* in matched serum samples might represent a promising biomarker for breast cancer with ease of access to analysis.

MATERIALS AND METHODS

P14^{ARF}/*MDM2*/*TP53* and *PTEN* pathway analysis

Pathway analysis was accessed by Pathway Studio® software program version 7 and the ResNet® 7 (Mammal) database (Ariadne Genomics, Inc., Rockville, USA). Four candidate genes (*P14^{ARF}*, *MDM2*, *TP53* and *PTEN*) were analyzed in depth by this software to increase the biological perspective of this study and facilitate the understanding beyond their functional link to breast neoplasm (Fig. 1, Supplementary Material 1).

Sampling and pathological classification

DNA was isolated from 66 triple-matched samples (cancerous tissues, matched adjacent normal tissues and serum samples) from 22 breast cancer patients according to the standardized protocol as described previously (51,52). The study was approved by the local institutional review board (Ethic commission beider Basel and Sichuan University China). Written consent forms were collected from all patients who were involved in this study. All the blood samples were collected before delivering any therapeutic treatments.

Staging and grading was evaluated according to the WHO histological classification. According to pathological tumor type and immunohistochemistry staining, we separated our patient samples into two subgroups: invasive ductal carcinoma (IDC) and Infiltrating Lobular Carcinoma (ILC). Breast cancer characteristics, such as staging, histological grading and hormone receptor expression, of the patients are listed in Table 3.

Mutation analysis of *TP53*

Screening of *TP53* mutation has been performed on the DNA-binding domain (exons 5–8). Information on the primer sequences and PCR conditions used for sequence analysis are listed in Supplementary Material 2. Direct DNA sequencing was performed by using a Big Dye terminator v3.1 cycle sequencing kit and an automated sequencer (ABI 3130, Applied Biosystem). The results of DNA sequence analysis were compared with the reference sequences of

Table 3. Clinical characteristics of triple-matched patient samples

Breast cancer tumor type	Total no. of patients	Age mean ± S (range)	Pathologic stage		No. of patients with lymph node involvement	No. of patients with metastasis	Histological grade			ER-positive patients	PR-positive patients	C-ERB2-positive patients
			Early	Late			1	2	3			
Invasive ductal carcinoma (IDC)	16	51 ± 12.6 (32–77)	9	7	12	0	0	3	13	13	10	5
Infiltrating Lobular Carcinoma (ILC)	6	48 ± 10.4 (37–61)	2	4	3	0	0	3	3	5	1	3

GenBank (www.ncbi.nlm.mih.gov/nucleotide) using DNASTAR sequence alignment software (DNASTAR Lasergene 8, Inc., Madison, USA). All sequencing reactions were performed in both directions and confirmed for concordance.

Methylation analysis of *P14^{ARF}*/*MDM2*/*TP53* and *PTEN* pathways using thymidine-specific cleavage mass array on MALDI-TOF MS

The SEQUENOM's EpiTYPER™ assay is a methylation quantification method which relies on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (53). In the current report, this assay was used to quantify the methylation of *TP53*, *P14^{ARF}*, *MDM2* and *PTEN* promoter regions.

The robustness of this approach for DNA methylation quantification has been confirmed by previous studies (32,52).

Bisulfite treatment. Bisulfite conversion of the target sequences was performed according to the instructions of a commercial Epitect® Bisulfite kit (QIAGEN AG, Basel, Switzerland).

Primer designing and PCR tagging for EpiTYPER™ assay. To design PCR primer for the candidate genes, CpG density and CpG sites of four target sequences were analyzed. Primer pairs have been designed to cover the promoter regions with the most CpG sites using MethPrimer (54). In PCR amplification, a T7 promoter tag was added to the reverse primer, and a 10mer tag sequence was added to the forward primer to balance the PCR primer length. Ensuring that *PTEN* primers discriminate between the gene and pseudogene (*psiPTEN*), the reverse primer was designed in a promoter region consisting of different nucleotides compared with the *psiPTEN* sequence (AL356489 and AF029308) especially at the 3′ end of the primer. The primer sequences, annealing temperatures (T_a) and PCR conditions are described in Supplementary Material 2.

In vitro transcription, T-cleavage assay and mass spectrometry. Unincorporated dNTPs were dephosphorylated by adding 1.7 μl of H_2O and 0.3 U of shrimp alkaline phosphatase (SAP; SEQUENOM, Inc., San Diego, CA, USA). The reaction mixture was incubated at 37°C for 20 min, and the SAP was then heat inactivated for 10 min at 85°C. Typically, 2 μl of the PCR were directly used as a template in a 5 μl transcription reaction. Twenty units of T7 RNA polymerase and DNA polymerase (Epicentre, Madison, WI, USA) were used to incorporate dTTP in to the transcripts. Ribonucleotides were used at 1 mmol/l and the dNTP substrate at 2.5 mmol/l. In the same step, the *in vitro* transcription RNase A (SEQUENOM) was added to cleave the *in vitro* transcript (T-cleavage assay). The mixture was further diluted with H_2O to a final volume of 27 μl. Twenty-two nanoliters of cleavage reaction were robotically dispensed (nanodispenser) onto silicon chips preloaded with matrix (SpectroCHIP; SEQUENOM). Mass spectra were collected using a MassARRAY Compact MALDI-TOF (SEQUENOM) and the spectra's methylation proportions were generated by the Epityper software v1.0 (SEQUENOM).

mtDNA alteration analysis

Mutation analysis of the mtDNA D-loop region. Mutation in the D-loop region of mtDNA was analyzed according to a previously published method (43). Information on primers and PCR conditions used for sequence analysis are listed in Supplementary Material 2. The results of DNA sequencing were analyzed as described before. Nucleotide numbering was based on the current mutation nomenclature recommendations (www.hgvs.org/mutnomen). Somatic mutations were scored as any difference between tumor and normal tissues. We considered this to be germline variation when it was found in both the tumor and normal tissues but different from the GenBank data base.

Quantity assessment of mtDNA and data collection. The mtDNA content was measured by multiplex quantitative PCR (q-PCR) using the mtDNA-encoded ATPase 8 (MTATP 8, starting at locus 8446) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) reference gene. The efficiency of the multiplex synchronized quantification of both *GAPDH* and *MTATP 8* was measured in the previous study of our group using standard curves obtained by serial dilution (40). The sequence and information on the primers and probe for the *GAPDH* and *MTATP 8* reference genes and the q-PCR conditions are summarized in Supplementary Material 2.

Q-PCR was carried out using the ABI PRISM 7000 sequence detection system in 25 μl of total reaction volume containing 7 μl of H_2O, 12.5 μl of PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 0.75 μl of each of the above-mentioned 10 μM primers (Microsynth, Balagach, Switzerland), 1 μl of a 5 μM FAM-labeled MTATP 8 probe and 0.5 μl of a 5 μM VIC-labeled GAPDH probe (both probes from Applied Biosystems) and for each reaction 1 μl of DNA was added.

Each sample was measured in duplicate and one negative control was included in every run.

Standard curves with known genomic DNA concentrations ranging from 3.125×10^4 to 10 pg/μl with six serial dilutions (i.e. 31 250, 6250, 1250, 250, 50 and 10 pg/μl) were performed. The threshold cycle (Ct) values of *GAPDH* and *MTATP* 8 were obtained by ABI Prism 7000 SDS software. The standard curves had average slopes at approximately −3.3 (~100% efficiency) and R^2 was >0.98. The content of mtDNA was calculated using the delta Ct (ΔCt) of an average Ct of mtDNA and nDNA (ΔCt = CtnDNA − CtmtDNA) in the same well as an exponent of 2 ($2^{\Delta Ct}$) (55).

Statistical methods

Data analysis was performed using the SPSS software (Statistical Software Package for Windows, version 17). Distribution of data was analyzed by Kolmogorov–Smirnov test that demonstrated our data set was not normally distributed ($P < 0.001$). The quantitative methylation profile of the four genes was compared between cancerous tissues, matched normal tissues and serum samples using two-way hierarchical cluster analysis. The most variable CpG sites for each gene were clustered based on pair-wise Euclidean distances and a linkage algorithm for all studied samples according to the

previously developed method by Gene Expression Statistical System (GESS) version 7.1.19 (NCSS, Kaysville, UT, USA) (32,52,56) followed by Mann–Whitney *U*-test.

The Mann–Whitney *U*-test and non-parametric Spearman's rho test were performed to determine the significance and correlation of mtDNA content analysis, D-loop mutations and clinical–pathological parameters.

Additional information

Cell signaling and pathway analysis data are available in Supplementary Material 1. The sequence of PCR primers and PCR conditions are available in Supplementary Material 2. The complete data for high-throughput methylation analysis of informative CpG sites in four breast cancer-related genes, including gene location, amplicon size and two-way hierarchical cluster analysis, are shown in Supplementary Material 3. Correlation between promoter methylation, D-loop mutations and mtDNA content with clinical parameters is given in Supplementary Material 4.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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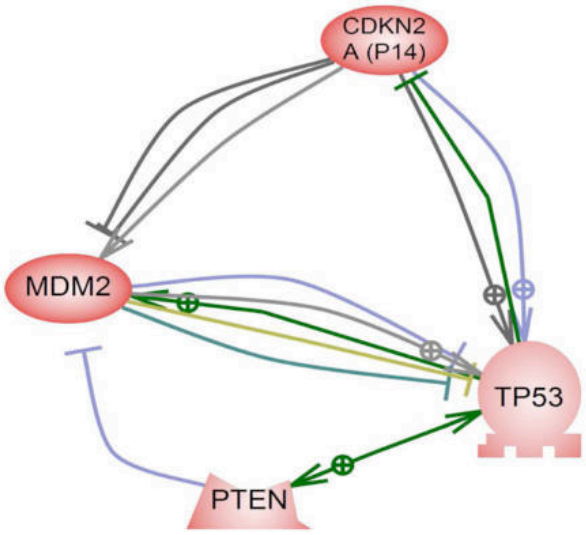
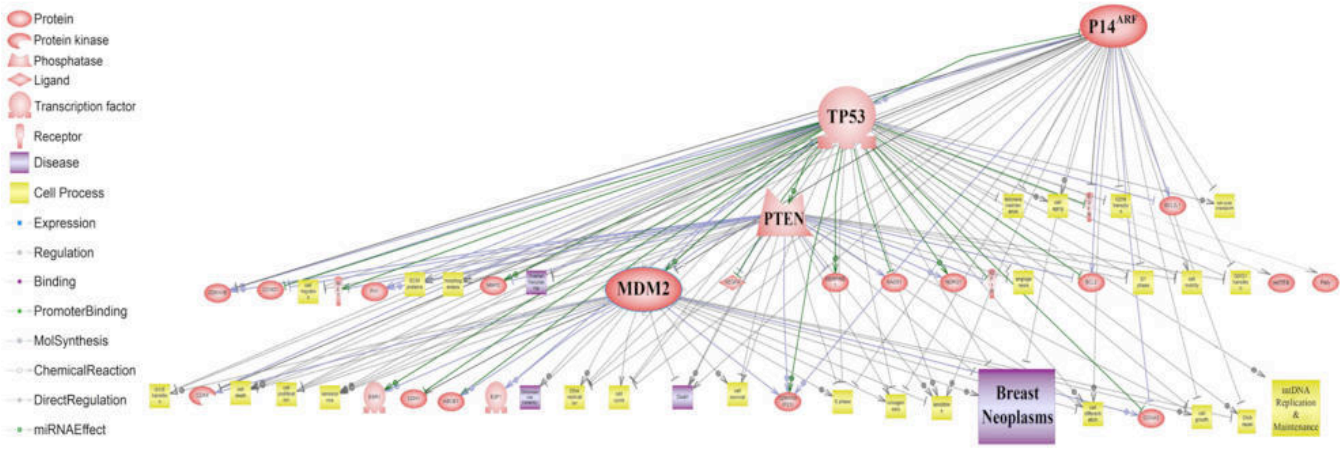
Supplementary Data 1



Direct force analysis of the four candidate genes.



Hierarchical Pathway analysis of candidate genes according to their effect on breast carcinogenesis.

Supplementary Data 2

Table 1. The sequence of PCR primers for DNA sequencing

Gene		Primer	Sequence (5'→3')	Length	T_a	Prodoct Size (bp)
TP53	Exon-5	Forward	GTTTCTTTGCTGCCGTCTTC	20	58.00	365
		Reverse	AGCCCTGTCGTCTCTCCAG	19		
	Exon-6	Forward	GAGACGACAGGGCTGGTTG	19	59.00	213
		Reverse	TAACCCCTCCTCCCAGAGAC	20		
	Exon-7	Forward	CCTGCTTGCCACAGGTCTC	19	58.00	271
		Reverse	GTATGGAAGAAATCGGTAAG	20		
	Exon-8	Forward	GCCTCTTGCTTCTCTTTTCC	20	58.00	329
		Reverse	TAACTGCACCCTTGGTCTCC	20		
mtDNA	D-Loop	Forward	CCCCATGCTTACAAGCAAGT	20	58.00	982
		Reverse	GCTTTGAGGAGGTAAGCTAC	20		

The PCR for each exon of P53 and D-loop region carried out in a 50 µl total volume containing 100ng DNA, 200µM of each dNTP, 20 pmol of each primer, 2.5 U PfuUltra high-fidelity DNA polymerase (Stratagene,La Jolla, CA), and 1X PfuUltra HF reaction buffer (for D-loop) or 2.5 U Hot Start Taq DNA polymerase, 1X PCR buffer and 1.5 mM MgCl2 (for P53 exons). The PCR was performed in 35 cycles under following condition: denaturation at 94°C for 15 sec, annealing step for 15 sec at appropriate T_a that is mentioned above, and 40 sec (for P53) or 90 sec (for D-loop) primer extension at 72°C.

Table 2. The sequence of PCR primers for Real-time PCR

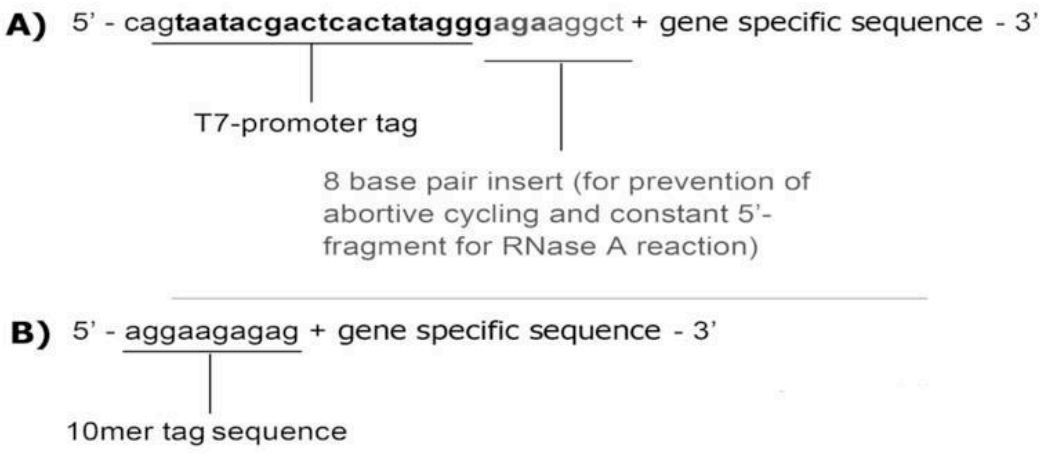
Gene	Primer	Sequence (5'→3')	Length	T_a	Prodoct Size (bp)
GAPDH	Forward	CCCCACACACATGCACTTACC	21	60	97
	Reverse	CCTAGTCCCAGGGCTTTGATT	21		
	Probe	MGB–TAGGAAGGACAGGCAAC–FAM	17		
MTATP 8	Forward	AATATTAAACACAAACTACCACCTACC	27	60	79
	Reverse	TGGTTCTCAGGGTTTGTTATA	21		
	Probe	FAM-CCTCACCAAAGCCCATA-MGB	17		

The multiplex quantitative PCR was performed using ABI PRISM 7000 sequence detection system (Applied Biosystems, Branchburg, New Jersey, USA) under the following conditions: an initiation step for 2 minutes at 50°C, followed by an initial denaturation step at 95°C for 10 minutes and a further step consisting of 40 cycles of 15 seconds at 95°C and one minute at 60°C.

Table 3. The sequence of PCR tagged primers for in vitro transcription and quantitative methylation study

Gene	Primer	Sequence (5'→3')	Length	T_a	Product Size (bp)
MDM2	tag-EN1-FW	AGGAAGAGAGAAGGAGTGTTATAGYGTTAAATTTTG	26+10	58	303
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCCACACAAACCCCAAAAA	18+31		
$P14^{ARF}$	tag-EN1-FW	AGGAAGAGAGGTTTTTGGTAGGGTYGTGTT	20+10	58	425
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTACTCCTACCCCCTTAACTACAAA	23+31		
PTEN	tag-EN1-FW	AGGAAGAGAG GAGTGGGAATTTGGAAAGTTTTTTA	25+10	58	451
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCAAAAACCCAAAAAACACCTATCT	24+31		
TP53	tag-EN1-FW	AGGAAGAGAGATGGTTTYGAAGTTTTTAGGGAT	23+10	58	449
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTAATACAAAACCTACTACRCCCTCT	24+31		

For the PCR on bisulphite-treated genomic DNA (gDNA) was performed in 40 cycles under following conditions: denaturation at 95°C for 20s, annealing step for 30s, primer extension at 72°C for 1 min .
The PCR cocktail was: 20 ng DNA, 1pmol of each primer, 200µM dNTP, 0.2 U Hot Start Taq DNA polymerase, 1.5 mM MgCl2 and the buffer supplied with the enzyme.



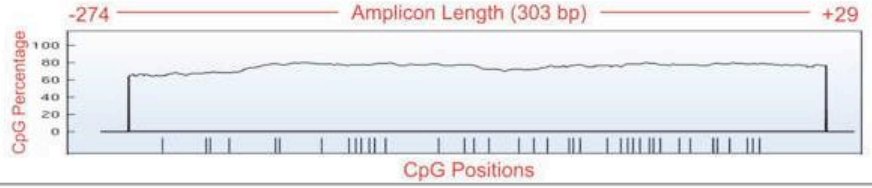
Primer taging for in vitro transcription. (A) Reverse primer with T7-promoter tag. (B) Forward primer with 10mer tag sequence as balance.

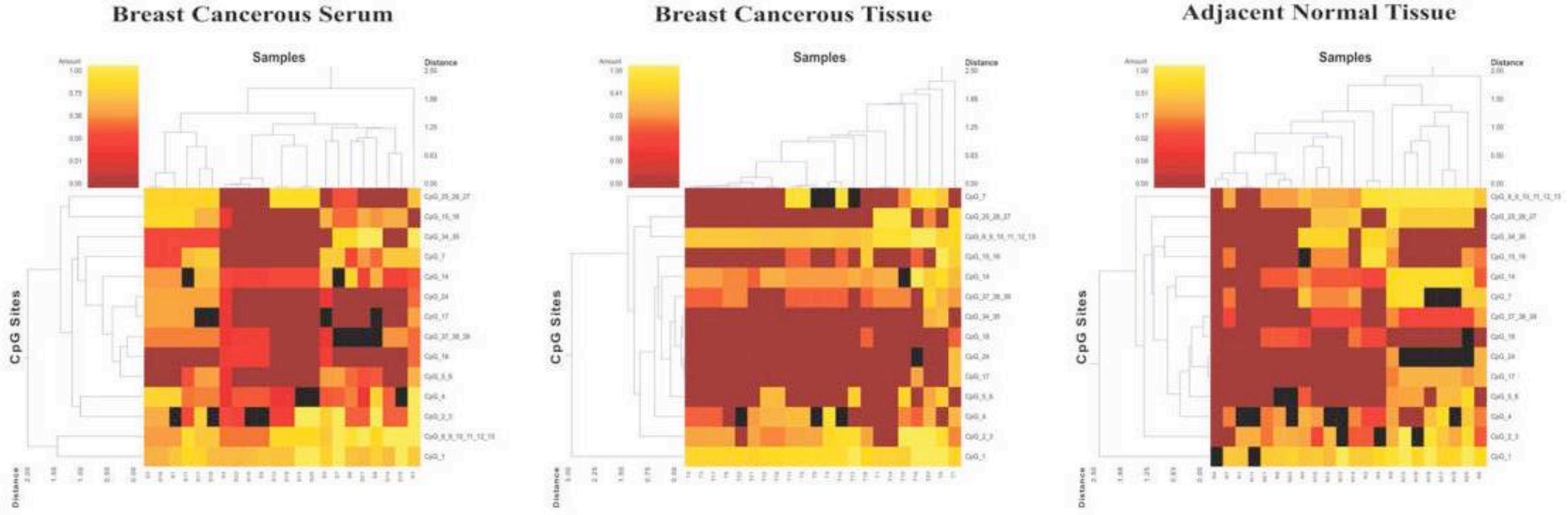
Supplementary Data 3

High-throughput methylation analysis of informative CpG sites in four genes related to breast cancer.

MDM2 Gene

Gene ID	locus	Function	Methylation effect on breast cancer
4193	12q14.3-q15	This gene is a target gene of the transcription factor tumor protein p53. The encoded protein is a nuclear phosphoprotein that binds and inhibits transactivation by tumor protein p53. Overexpression of this gene can result in excessive inactivation of p53.	direct

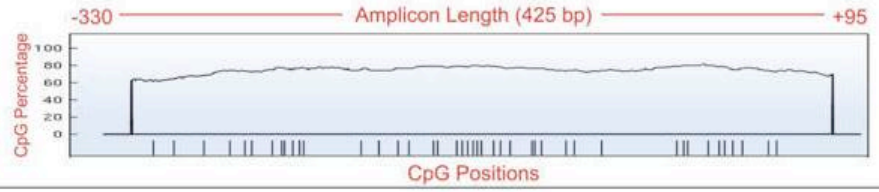


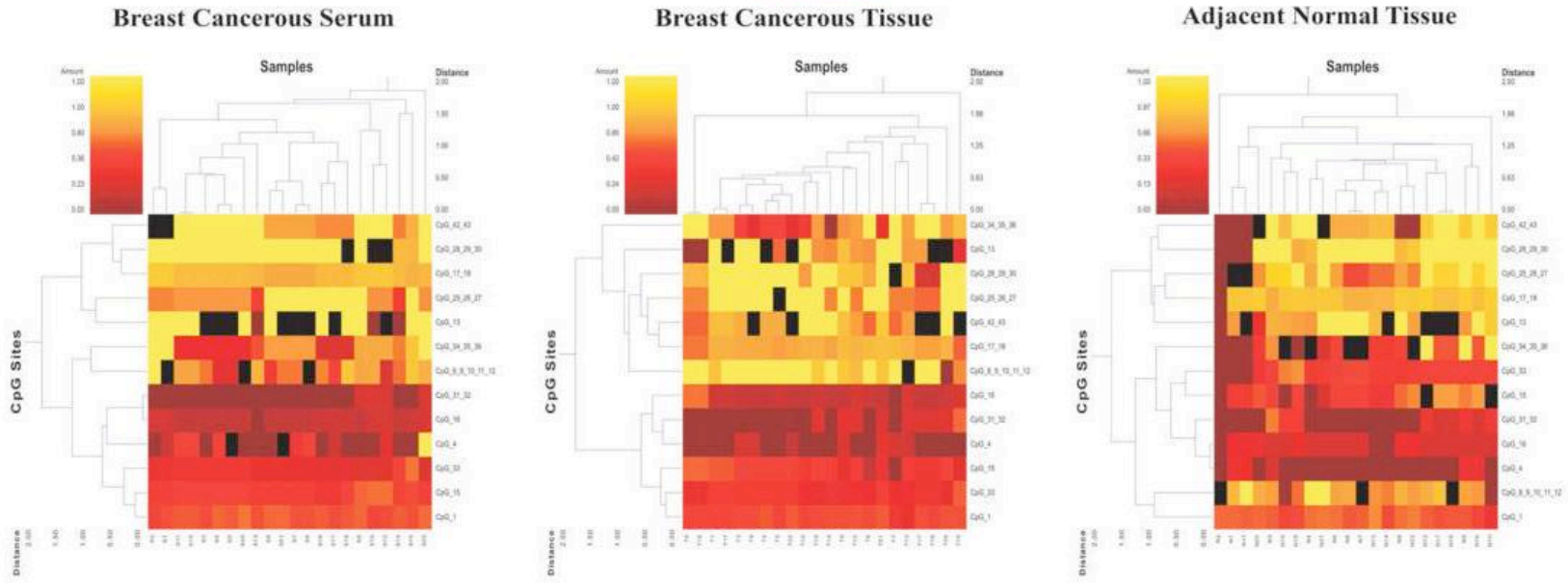


Double dendrogram of MDM2 gene: Two-way hierarchical cluster analysis of triple samples from 66 (breast cancerous tissues, matched serum samples and normal tissues) of 22 breast cancer patients. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

$P14^{ARF}$ Gene

Gene ID	locus	Function	Methylation effect on breast cancer
1029	9p21	Cell cycle regulation, involved in senescenc: This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53.	direct

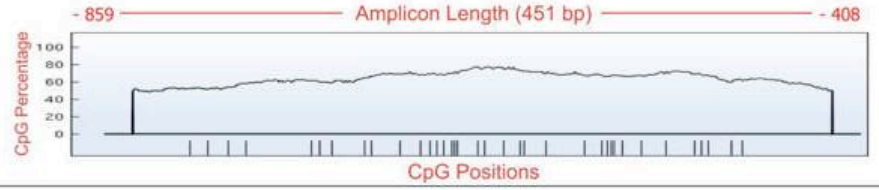


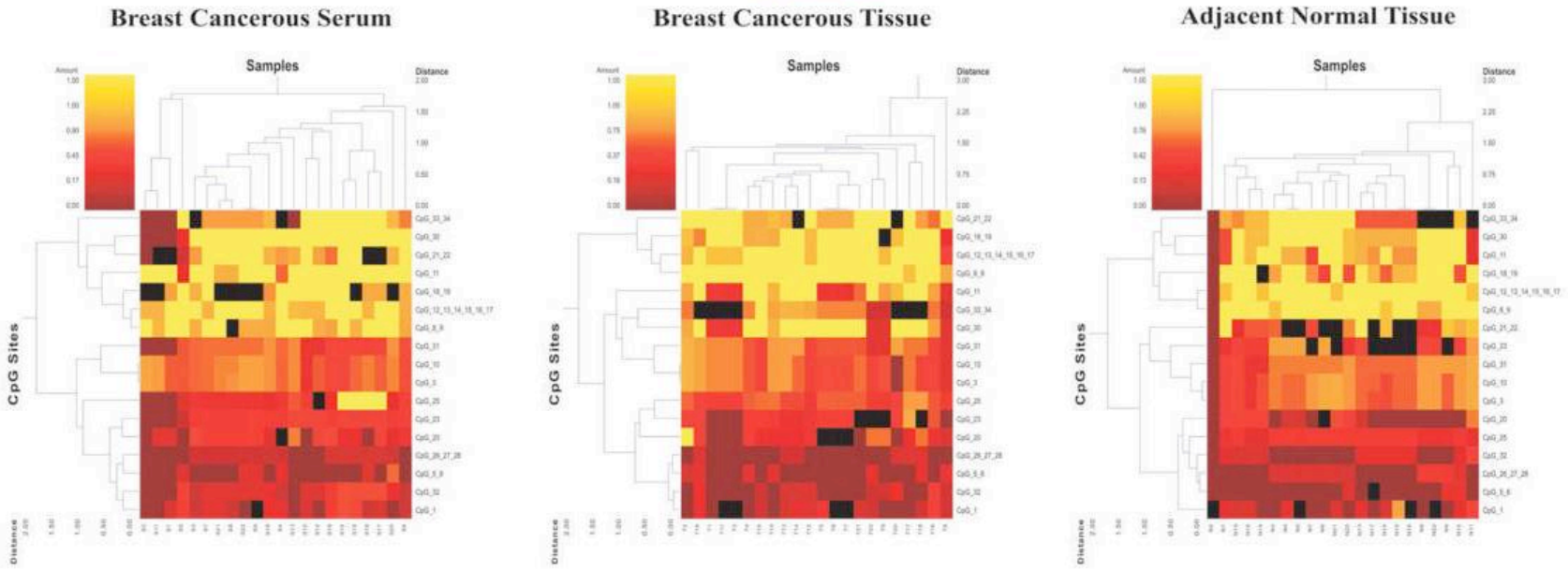


Double dendrogram of $P14^{ARF}$ gene: Two-way hierarchical cluster analysis of triple samples from 66 (breast cancerous tissues, matched serum samples and normal tissues) of 22 breast cancer patients. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

PTEN Gene

Gene ID	locus	Function	Methylation effect on breast cancer
5728	10q23.3	This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase.	direct

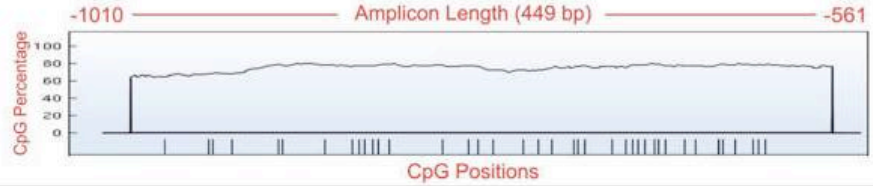


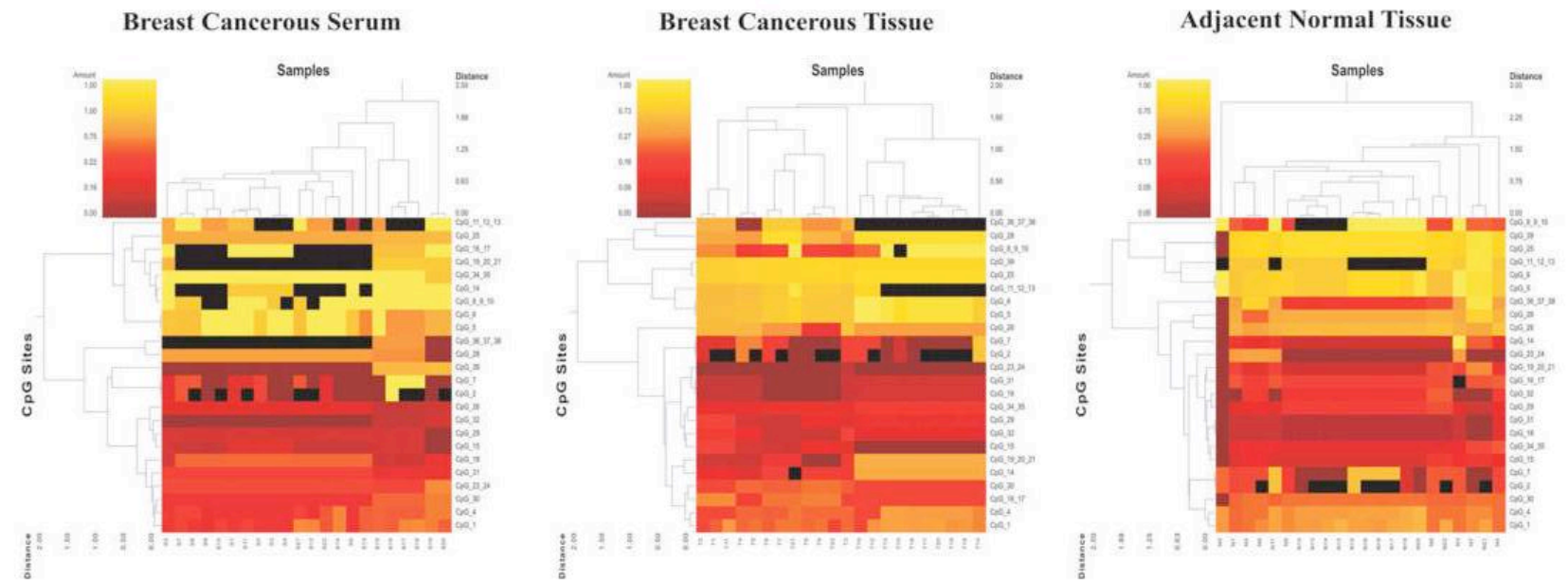


Double dendrogram of PTEN gene: Two-way hierarchical cluster analysis of triple samples from 66 (breast cancerous tissues, matched serum samples and normal tissues) of 22 breast cancer patients. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

TP53 Gene

Gene ID	locus	Function	Methylation effect on breast cancer
7157	17p13.1	Apoptosis, cell cycle regulation, inhibition of growth and invasion.	direct





Double dendrogram of TP53 gene: Two-way hierarchical cluster analysis of triple samples from 66 (breast cancerous tissues, matched serum samples and normal tissues) of 22 breast cancer patients. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

3.4 Published research article:

Methylation Signature of Lymph Node Metastases in Breast Cancer Patients

Author: Zeinab Barekati, Ramin Radpour, Qing Lu, Johannes Bitzer, Hong Zheng, Paolo Toniolo, Per Lenner, Xiao Yan Zhong

Journal: BMC Cancer

Summary: Invasion and metastasis are important hallmarks of malignant tumours caused by complex genetic and epigenetic alterations. DNA methylation profile of twelve cancer related genes were assessed to identify a potential metastatic signature of them. These genes were selected from the previous studies in our group. The results of the present study showed methylation heterogeneity between primary tumors and metastatic lesion. The contribution of aberrant methylation alterations of *BMP6, BRCA1* and *P16* genes in metastatic lymph nodes suggests implementing these genes as markers for screening metastasis.

Author contributions: Zeinab Barekati was involved in experimental design, performing the study, data analysis, interoperating data and writing the manuscript.

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BMC Cancer

RESEARH ARTICLE Open Access

Methylation signature of lymph node metastases in breast cancer patients

Zeinab Barekati[1], Ramin Radpour[1], Qing Lu[2], Johannes Bitzer[3], Hong Zheng[4,5], Paolo Toniolo[6], Per Lenner[7] and Xiao Yan Zhong[1*]

Abstract

Background: Invasion and metastasis are two important hallmarks of malignant tumors caused by complex genetic and epigenetic alterations. The present study investigated the contribution of aberrant methylation profiles of cancer related genes, *APC, BIN1, BMP6, BRCA1, CST6, ESR-b, GSTP1, P14 (ARF), P16 (CDKN2A), P21 (CDKN1A), PTEN,* and *TIMP3*, in the matched axillary lymph node metastasis in comparison to the primary tumor tissue and the adjacent normal tissue from the same breast cancer patients to identify the potential of candidate genes methylation as metastatic markers.

Methods: The quantitative methylation analysis was performed using the SEQUENOM's EpiTYPER™ assay which relies on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Results: The quantitative DNA methylation analysis of the candidate genes showed higher methylation proportion in the primary tumor tissue than that of the matched normal tissue and the differences were significant for the *APC, BIN1, BMP6, BRCA1, CST6, ESR-b, P16, PTEN* and *TIMP3* promoter regions ($P<0.05$). Among those candidate methylated genes, *APC, BMP6, BRCA1* and *P16* displayed higher methylation proportion in the matched lymph node metastasis than that found in the normal tissue ($P<0.05$). The pathway analysis revealed that *BMP6, BRCA1* and *P16* have a role in prevention of neoplasm metastasis.

Conclusions: The results of the present study showed methylation heterogeneity between primary tumors and metastatic lesion. The contribution of aberrant methylation alterations of *BMP6, BRCA1* and *P16* genes in lymph node metastasis might provide a further clue to establish useful biomarkers for screening metastasis.

Keywords: Methylation, Metastasis, Breast cancer, Biomarker

Background

Breast cancer is one of the most common malignancies with a high mortality rate among women [1]. Breast cancer, a heterogeneous disease, presents various pathological signs such as axillary lymph node metastasis which is associated with a high risk of recurrence and considered as an important prognosis factor in the early stages of the disease [2,3]. Invasion and metastasis are two important hallmarks of malignant tumors associated with complex genetic and epigenetic alterations that allow tumors to disseminate throughout lymphatics or blood vessels, giving rise to the colonization and growth of metastatic cells in distant organs [4-6]. Considering that tumor dissemination is an early event in breast cancer [7], genetic and epigenetic analysis of tumors and metastatic lesions could provide results for biomarker discovery and may improve diagnosis, prognosis and proper management of the treatment for breast cancer patients.

The contribution of aberrant DNA hypermethylation of cancer related genes to the transcriptional silencing and carcinogenesis has been demonstrated in different diseases including different cancer types [7,8]. The methylation profile of genes involved in critical molecular processes such as cell cycle control, DNA repair and angiogenesis in breast cancer has been investigated [9-12]. Since the

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lymphatic system has a direct dispatch to spread primary tumor cells to the lymph nodes in breast cancer, primary tumor signatures has been considered as surrogate for lymph node metastasis. However, this persuasion has recently been controversial especially in the context of DNA methylation pattern from primary to metastasis in breast cancer [9,10,13]. According to best of our knowledge genome-wide DNA methylation was reported in few metastatic breast cancer cell lines ,however, methylation pattern of individual candidate genes in both the primary and lymph node metastases has been explored in clinical specimens [14,15]. Therefore the profile of metastasis in breast cancer patients is less characterized and poorly understood which needs further studies to understand the relationship between epigenetic alteration and metastases in breast cancer.

Our previous approach revealed methylation signatures of 42, 528 CpG sites from 22 breast cancer candidate genes that demonstrated promoter hypermethylation of 10 genes involved in cell cycle and DNA repair, invasion and metastasis, cell proliferation, signal transduction and cell detoxification [16]. Moreover, we have shown hypermethylation of the two additional genes involved in *TP53* regulatory pathway in the breast cancer patients [17,18]. The significance of using these hypermethylated genes as circulating biomarkers has been explored as well [18]. The present study investigated the contribution of significant aberrant methylation profile of twelve cancer related genes from the aforementioned studies (*APC, BIN, BMP6, BRCA1, CST6, ESR-b, GSTP1, P14, P16, P21, PTEN* and *TIMP3)* in matched axillary lymph node metastasis in comparison to the primary tumor tissue and the adjacent normal tissue from the same breast cancer patients to identify the potential of aberrant methylation profile of the candidate genes as metastatic signature.

Methods

Sampling and pathological classification

The study was approved by the local institutional review board (Ethic commission beider Basel, Sichuan University China). Written consent forms were collected from all patients who were involved in this study. Staging and grading was evaluated according to the WHO histological classification. DNA was isolated from 65 samples including matched primary tumors tissue, matched normal tissue and their matched lymph node metastasis of 24 chines patients with breast cancer. The present cohort included 17 matched normal breast tissues that were collected at least 4 cm away from the tumor site and were confirmed as normal tissue by pathologist. The axillary lymph nodes were removed at the same surgery. Part of the samples was embedded using OCT (Optimal Cutting Temperature, Sakura Finetek, U.S.A) and stored in liquid nitrogen. The above procedures were completed within 20 minutes after peeling preparation. The samples were then stored at -80°. The frozen tissues were sectioned in 4 μm thickness and were submitted for hematotoxylin and eosin staining study. According to pathological tumor type and immunohistochemistry staining, studied cohort consisted of patient's with Invasive Ductal Carcinoma (IDC) and Invasive Lobular Carcinoma (ILC). Breast cancer characteristics, such as staging, histological grading, and hormone receptor expression from the breast cancer patients are listed in Table 1.

The entirely neoplastic and adjacent normal frozen sections were subjected for DNA extraction. Five to ten sections with 90% neoplastic coverage applied for DNA extraction, and last section was checked for the correct characterization, using the High Pure PCR Template Preparation Kit (Roche, Germany). The rest of the samples were fixed in 10% buffered formalin for immunohistochemical staining study.

The analysis for the detection of estrogen receptor (ER), progesterone receptor (PR) and HER2/neu (C-ErbB-2) proteins was carried out using primary antibodies for Rabbit anti-human estrogen receptor monoclonal antibody (clone sp1, Roche), Rabbit anti-human progesterone receptor monoclonal antibody (clone sp2, Roche), Rabbit anti-human HER-2/neu monoclonal antibody (clone 4B5, Roche). Then slide stainings were processed using ultraView Universal DAB Detection Kit (Ventana Medical Systems Inc, Tucson, AZ) (Figure 1). The ER, PR and HER-2/neu were analyzed following ASCO/CAP Guidelines.

Table 1 Clinical characteristics of the studied cohort

Total no. of patients	Breast cancer tumor type		Age mean ± S.D. (range)	Pathologic stage		No. of patients with distance metastasis	Histological grade			ER positive patients	PR positive patients	C-ERB2 positive patients
	IDC	ILC		Early (I,II)	Late (III)		1	2	3			
24	20	4	48 ± 9.53 (33-69)	14	10	0	1	5	18	19	12	12

IDC, Invasive Ductal Carcinoma; ILC, Invasive Lubular Carcinoma; ER, Estrogen Receptor; PR, Progestron Receptor.

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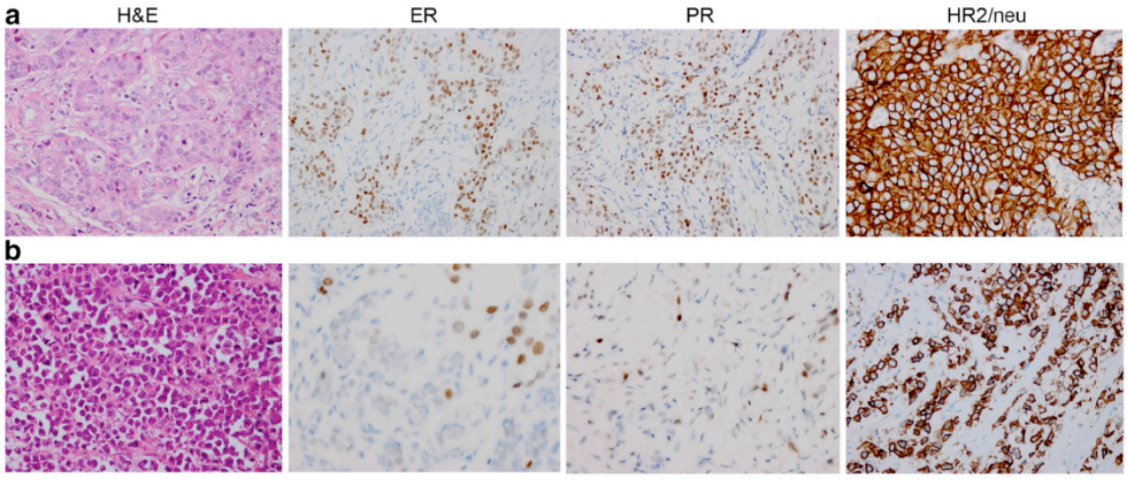

Figure 1 Hematotoxylin and eosin staining (H&E) and immunohistochemical staining for estrogen receptor (ER), progesterone receptor (PR) and HER2/neu proteins (400X). (a) Ductal carcinoma. **(b)** Lobular carcinoma.

Methylation analysis using thymidine-specific cleavage mass array on MALDI-TOF *silico-chip*

The SEQUENOM's EpiTYPER™ assay is a methylation quantification method which relies on MALDI-TOF MS [19]. The theory and practice of this approach for DNA methylation quantification has been confirmed by previous studies [16,20,21]. In the current study, this assay was used to quantify the methylation of *APC, BIN1, BRCA1, BMP6, CST6, ESR-b, GSTP1, P14, P16, P21, PTEN* and *TIMP3* promoter regions.

Bisulfite treatment

Bisulfite conversion of the target sequences was performed according to the instruction of the Epitect® Bisulfite Kit (QIAGEN AG, Basel, Switzerland).

Primer designing and PCR-tagging for EpiTYPER™ assay

To design PCR primer for the candidate genes, CpG density and CpG sites of the twelve targeted sequences were analyzed. According to our previous publications, we used the same primer sequences which were tagged with T7-promoter for the reverse and a 10 mer sequences to the forward primer to balance the PCR condition and primer pairs can cover the promoter regions with the most CpG sites using MethPrimer. The primer sequences, annealing temperatures (T_a) and PCR conditions are described in Additional file 1.

In vitro transcription, T-cleavage assay and mass spectrometry

Unincorporated dNTPs were dephosphorylated by adding 1.7μL H_2O and 0.3 units of shrimp alkaline phosphatase (SAP; SEQUENOM, Inc., San Diego, CA). The reaction mixture was incubated at 37°C for 20 minutes and the SAP was then heat inactivated for 10 minutes at 85°C. Typically, 2μL of the PCR were used directly as a template in a 5μL transcription reaction. Twenty units of T7 R&DNA polymerase (Epicentre, Madison, WI) were used to incorporate dTTP in the transcripts. Ribonucleotides were used at 1 mmol/L and the dNTP substrate at 2.5 mmol/L. In the same step, the *in vitro* transcription RNase A (SEQUENOM) was added to cleave the *in vitro* transcript (T-cleavage assay). The mixture was further diluted with H_2O to a final volume of 27μL. Twenty-two nanoliters of cleavage reaction were robotically dispensed (nanodispenser) onto silicon chips preloaded with matrix (SpectroCHIP; SEQUENOM, San Diego). Mass spectra data were collected using a MassARRAY Compact MALDI-TOF (SEQUENOM) and spectra's methylation proportions were generated by the Epityper software v1.0 (SEQUENOM, San Diego).

Cell signaling and pathway analysis

Gene networks and canonical pathways displaying hypermethylated genes in lymph node metastasis were identified using the Pathway Studio® software version 7.1 (Mammal) database (Ariadne Genomics, Inc., Rockville, USA). The functional analysis identified the biological perspective of the genes that were most relevant to the data sets and facilitated the understanding beyond their functional link to breast neoplasm and metastasis.

Statistical methods

Data analysis was performed using the SPSS software (Statistical Software Package for Windows, version 19).

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Distribution of data was analyzed by Kolmogorov-Smirnov test that demonstrated our data set was not normally distributed ($P < 0.001$). Quantitative methylation profile of the twelve candidate genes were compared among primary tissue, adjacent matched normal tissue and their matched lymph node metastasis using the two-way hierarchical cluster analysis. The CpG sites for each gene were clustered based on pair-wise Euclidean distances and linkage algorithm for all studied samples according to the previously developed method by Gene Expression Statistical System (GESS) version 7.1.19 (NCSS, Kaysville, Utah, USA) and the statistical differences between mean methylation quantity of the informative CpG sites per genes were identified using Mann-Whitney-U-Test. The non-parametric Spearman's rho test was performed to find out significance clinical-pathological parameters.

Results

Quantitative methylation profiles of the 12 breast cancer candidate genes promoter using MALDI-TOF MS

The methylation profiles of 12 breast cancer candidate genes in matched primary tumors, normal tissue and their matched lymph node metastasis from 24 breast cancer patients were assessed. For all the genes, one amplicon per gene and 250 CpG sites in total per sample (total of 16,250 sites in 65 analyzed samples) were analyzed (Table 2; Additional file 2.)

The different levels of methylation between studied samples were identified using the two-way hierarchical cluster analysis. The methylation cluster analysis for each individual studied gene is illustrated in Additional file 2.

Methylation alterations of the primary tumor tissue

Hierarchical cluster analysis profiling of the promoter alterations and the significance of the alterations are shown in Figure 2(a) for the 12 studied genes.

Methylation quantification of the 12 candidate genes revealed significantly higher methylation proportion for promoter regions of *APC, BIN1, BMP6, BRCA1, CST6, ESR-b, P16, PTEN* and *TIMP3* in primary tumor tissue versus matched normal tissue ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.0001$, $P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.01$; respectively). Methylation analysis of *GSTP1, P14* and *P21* in primary tumor tissue showed slightly higher methylation proportion in comparison to the matched normal tissue, however, the differences were not significant (Figure 2b).

Methylation alterations of the matched lymph node metastasis

Comparison of methylation profiles of the 12 breast cancer candidate genes in the paired lymph node metastasis to the matched normal tissue showed significantly higher methylation levels for *APC, BMP6, BRCA1* and *P16* genes ($P < 0.05$ and $P < 0.01$, $P < 0.0001$, $P < 0.05$; respectively). The lymph node metastasis was even more hypermethylated for *BMP6* than the primary tumor tissue ($P < 0.05$). The promoter region of *BIN1, BMP6, BRCA1, GSTP1, P14* and *TIMP3* showed significantly lower methylation ratio in the lymph node metastasis

Table 2 High-throughput methylation analysis of CpG sites per amplicon for the 12 candidate genes

Genes	Amplicon size (bp)	Total No. of CpG sites in amplicon	No. of analyzed CpG sites in amplicon	No. of analyzed CpG sites per amplicons	
				Single sites	Composite sites
APC	420	26	11	9	2
BIN1	330	32	12	3	9
BMP6	397	37	26	9	17
BRCA1	413	30	15	10	5
CST6	445	49	19	11	8
ESR-b (ER beta)	374	30	13	6	7
GSTP1	381	23	16	11	5
P14	425	36	22	5	17
P16 (CDKN2A)	580	62	38	12	26
P21 (CDKN1A)	419	30	9	8	1
PTEN	451	34	29	10	19
TIMP3v	441	51	40	12	38

The *in silico* digestion was performed for the T-cleavage assay. The percentage of total CpG sites in the amplicon is divided into single sites (single CpG sites) and composite sites (two or more adjacent CpG sites fall within one fragment, or when fragment masses are overlapping).

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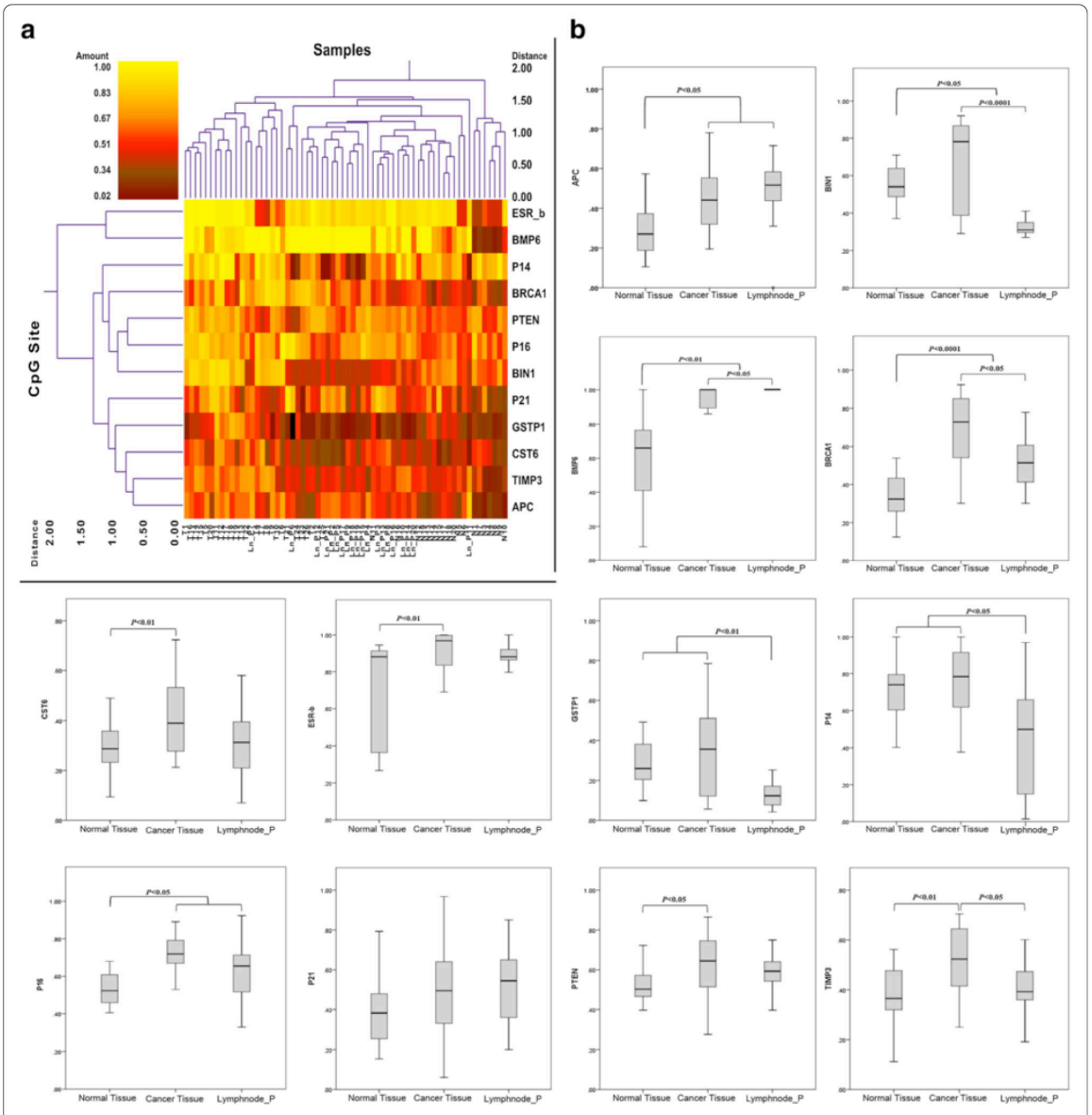

Figure 2 a) **Double dendrogram presents the methylation profiles of the twelve candidate genes in tumor tissue, matched normal tissue and lymph nodes metastasis (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100). b)** Comparison between quantitative analysis (0%-100%) of methylation for the candidate genes in the studied cohort (* significant correlation; Mann-Whitney U Test).

than the primary tissue ($P < 0.0001$, $P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.05$, $P < 0.05$; respectively).

Comparing the lymph node metastasis to the matched normal tissue, the former revealed differentially lower methylation ratio for *BIN1, GSTP1* and *P14* promoter regions ($P < 0.05$, $P < 0.01$, $P < 0.05$; respectively) (Figure 2).

Relationship of methylation alterations within the promoter region of 12 breast cancer candidate genes and clinicopathological parameters

Clinicopathological parameters with the methylation proportion of the 12 candidate genes in primary tumor tissue and matched lymph node metastasis were analyzed. The analyses showed significant correlation between higher

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methylation level of *GSTP1* and increasing the histological grade in the primary tumor tissue (Spearman's rho test; $P < 0.05$). The higher methylation proportion of *BIN1* showed significant correlation with expressed ER in primary tumor tissue (Spearman's rho test; $P < 0.01$). Methylated *TIMP3* revealed significant correlation to the primary tumor tissue lacking expression of PR (Spearman's rho test; $P < 0.05$). The increase of methylation of *P21* showed an inverse correlation with the expression of C-ERB2 in the primary tumor tissue (Spearman's rho test; $P < 0.05$). We could not find any significant correlations between methylation alterations in either of the matched lymph node metastasis and clinicopathological parameters.

Discussion

Aberrant methylation profiles and silencing of a small subset of tumor suppressors and cancer related genes involved in both the primary tumor and lymph node metastasis has been investigated for breast cancer [11,22-24]. Several studies on breast cancer revealed some similarities and differences between promoter methylation pattern of the studied genes in primary and lymph node metastatic [16,19,25]. These investigations highlight the important role of aberrant promoters' methylation in the metastatic process. In the present study, we implemented the methylation signature of the 12 breast cancer candidate genes (*APC, BIN1, BMP6, BRCA1, CST6, ESR-b, GSTP1, P14, P16, P21, PTEN* and *TIMP3*) by comparing lymph nodes metastasis to their matched primary tumor tissues and normal tissues from the same breast cancer patients.

The quantitative methylation analysis of the 12 studied genes in the present cohort showed higher methylation proportion for the primary tumor tissue versus matched normal tissue and the differences were significant for *APC, BIN1, BMP6, BRCA1, CST6, ESR-b, P16, PTEN* and *TIMP3* promoter regions ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.0001$, $P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.01$; respectively). Among the significant methylated genes, *APC, BMP6, BRCA1* and *P16* represented higher methylation proportions in matched lymph node metastasis compared to those of the normal tissue ($P < 0.05$ and $P < 0.01$, $P < 0.0001$, $P < 0.05$; respectively). Present findings provided evidence of differences in methylation status between primary tumors and their corresponding matched lymph nodes and demonstrated methylation heterogeneity between primary tumors and metastatic lesion which are in line with previous reports described about primary tumor and metastasis in breast, gastric and colorectal cancers [13,26,27]. The results also indicated that some of the cancer specific changes become altered over the metastasis procedure. The mechanism for losing or gaining methylation in lymph nodes metastatic is still not clear while the alteration of the methylation signature from primary to metastasis might be due to the adaptation response of the disseminated cells to the microenvironment at the site of colonization [28].

The molecular function, biological processes and contribution of the studied genes to developing metastasis were analyzed by ResNet® 7 (Mammal). The pathway analysis revealed that *BMP6*, *BRCA1* and *P16* have a role in prevention of neoplasm metastasis (Figure 3). The relation of DNA methylation for *BRCA1* and *P16* with tumor recurrence has been reported with a high value in breast cancer patients [29]. Moreover, association of *P16*

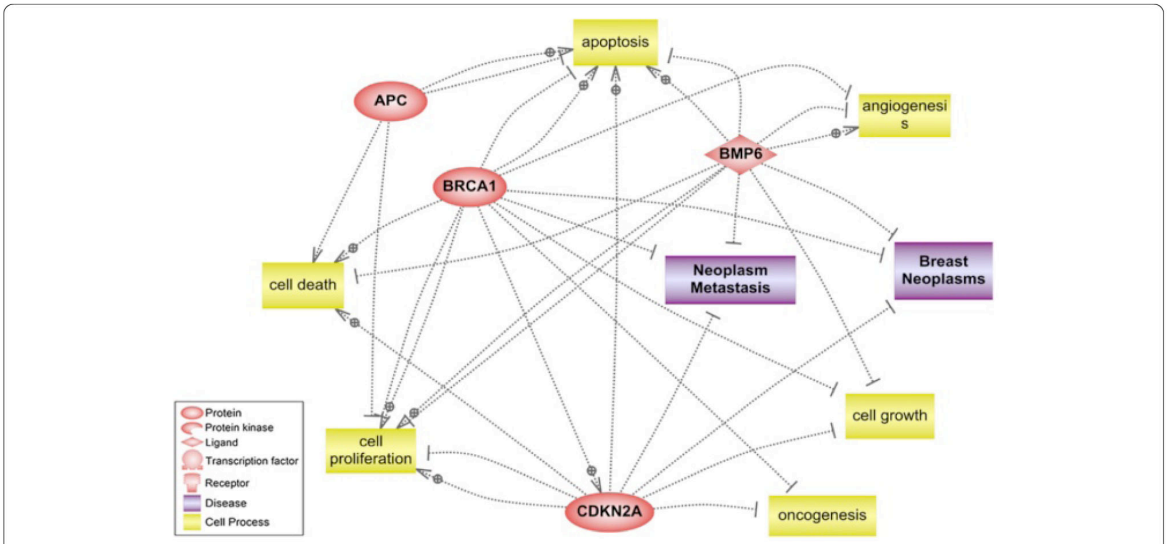

Figure 3 Pathway analysis of the hypermethylated genes in lymph node metastasis including their biological processes and association to breast carcinogenesis and neoplasm metastasis.

hypermethylation with cancer progression and lymph node invasion has been shown in different studies [30,31]. Then, the aberrant methylation signatures of these genes found in the metastatic lymph node can provide a further clue to establish useful biomarkers for screening metastasis in breast cancer. Such biomarkers will need to be additionally explored for the potential opposing functional effects of specific hypermethylated CpG sites on transcriptional activity as well as for absolute percent methylation cutoffs for each breast tumor type that would enable reliable utility of them in the clinical laboratory setting. The possibility of using these hypermethylated genes as biomarkers in our previous study was investigated with the aim of developing a blood based panel for plasma and serum samples of breast cancer patients [18]. Thus the detection of these three hypermethylated genes either as tissue specific biomarkers or as circulating biomarkers may give insight into the prognosis and therapeutic management of the breast cancer patients. Moreover, we identified significantly greater hypermethylation proportion of *BMP6* in the lymph node metastasis than their primary tumor tissue ($P < 0.05$). *BMP6* is a member of TGF-β super family and critically involved in many developmental processes [32]. Recently, the close association of *BMP6* with progression of tumorigenesis and regulation of invasion for tumor cells has been reported [33]. It has been assumed that demethylation of *BMP6* and re-expression of this gene might modulate metastasis and invasion in breast cancer [34,35].

Conclusions

In conclusion, the present study showed aberrant tumor-specific methylation alterations for *APC, BIN1, BMP6, BRCA1, CST6, ESR-b, GSTP1, P14, P16, P21, PTEN* and *TIMP3* in the studied cohort. Additionally, we identified methylation heterogeneity between primary tumors and metastatic lesions. The contribution of aberrant methylation alterations of *BMP6*, *BRCA1* and *P16* genes in lymph node metastasis might provide a further clue to establish useful biomarkers for screening metastasis, which might improve prognosis and therapeutic management of the breast cancer patients.

Additional files

Additional file 1: Supplementary Data 1. The sequence of PCR primers, PCR conditions and complete data for high-throughput methylation analysis of informative CpG sites in 12 breast cancer-related genes, including: gene location, amplicon size and two-way hierarchical cluster analysis are illustrated in dataset.

Additional file 2: Supplementary Data 2. High-throughput methylation analysis of CpG sites for 12 candidate genes that are related to breast cancer.

Competing interest

There is no conflict of interest in the present study.

Authors' contributions

XYZ and ZB conceived and designed the experiment, ZB carried out experiments, ZB and RR analyzed the data, HZ and QL, provided samples, JB, PL, PT, XYZ material and analysis tools. ZB and XYZ were involved in writing the paper; all authors had final approval of the submitted and published versions.

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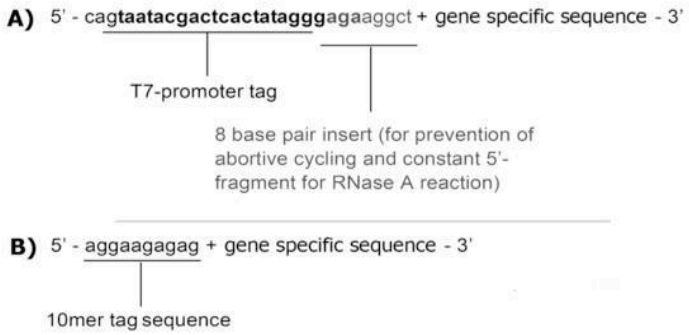
Supplementary Data 1

The sequence of PCR tagged primers for in vitro transcription and PCR conditions

For the PCR on bisulfite-treated genomic DNA (gDNA), the following PCR conditions were used: 1x: 95°C for 10 min; 48x: 95°C for 30s, Ta for 40s, 72°C for 1 min; 1x 72°C for 5 min. The PCR cocktail was: 2µL DNA (2.00µL of at least 10 ng/µL DNA for a final concentration of 2ng/µL per reaction) in a 10µL total volume using 1pmol of each primer, 200µM dNTP, 0.2 unit Hot Start Taq DNA polymerase, 1.5mM $MgCl_2$ and the buffer supplied with the enzyme.

For re-amplification, 2µL of first round PCR reaction (25–250ng amplicon) was directly used as template with the same primer pairs under the same PCR cycling conditions.

Gene	Primer	Sequence (5'→3')	Length	T_a	Prodoct Size (bp)
APC	tag-EN1-FW	AGGAAGAGAGATTGTTTTTTTGTGTTGTAAAAATTAT	27+10	58	420
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCACCTCCATTCTATCTCCAATAAC	24+31		
BIN1	tag-EN1-FW	AGGAAGAGAGGGAGGTGAGTTTTTGGAA	18+10	58	330
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCTACCTTTTAAAAAACCACTCC	22+31		
BMP6	tag-EN1-FW	AGGAAGAGAGGGGGTAAATTTTATGGTGGTTT	22+10	57	397
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCCTTCCTAACCCTCAATCCTTA	22+31		
BRCA1	tag-EN1-FW	AGGAAGAGAGAATTGGAGATTTTTATTAGG	20+10	56	413
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTAAATCTCAACRAACTCAC	18+31		
CST6	tag-EN1-FW	AGGAAGAGAGGTTGGTAGTTTATTTTGGATAGTTT	25+10	59	445
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCAAATCCCRAAATTCTCC	18+31		
ESR-b (ER beta)	tag-EN1-FW	AGGAAGAGAGTTTAGTTGTTGGTTTTTTGGAT	22+10	58	374
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTAAAATTTCAAACAAAATAAAACAATT	26+31		
GSTP1	tag-EN1-FW	AGGAAGAGAGTTYGGGAGGTTGAAGTAGA	19+10	60	381
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTAAACAAACAACAAAAAAAAAACC	23+31		
$P14^{ARF}$	tag-EN1-FW	AGGAAGAGAGGTTTTTGGTAGGGTYGTGTT	20+10	58	425
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTACTCCTACCCCCTTAACTACAAA	23+31		
P16 (CDKN2A)	tag-EN1-FW	AGGAAGAGAGGGTTGTTTTTGGTAGGG	17+10	58	580
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTATATAAACCACRAAAACCC	19+31		
P21 (CDKN1A)	tag-EN1-FW	AGGAAGAGAGGGTAAATTTTTGTTTGTTAGAGTGG	25+10	60	419
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTTAACTTCRACAACTACTCACACCT	24+31		
PTEN	tag-EN1-FW	AGGAAGAGAG GAGTGGGAATTTGGAAAGTTTTTTA	25+10	58	451
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCAAAAACCCAAAAAACACCTATCT	24+31		
TIMP3	tag-EN1-FW	AGGAAGAGAGTTTTGTTATTGGTTTGAGGG	20+10	59	441
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTCCAAACTCCAACTACCCA	18+31		



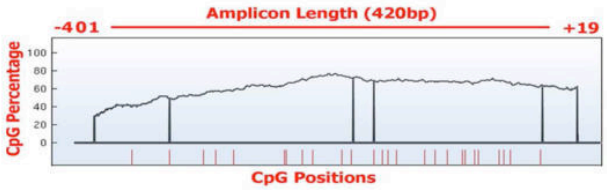
Primers for in vitro transcription. (A) Reverse primer with T7-promoter tag. (B) Forward primer with 10mer tag sequence as balance.

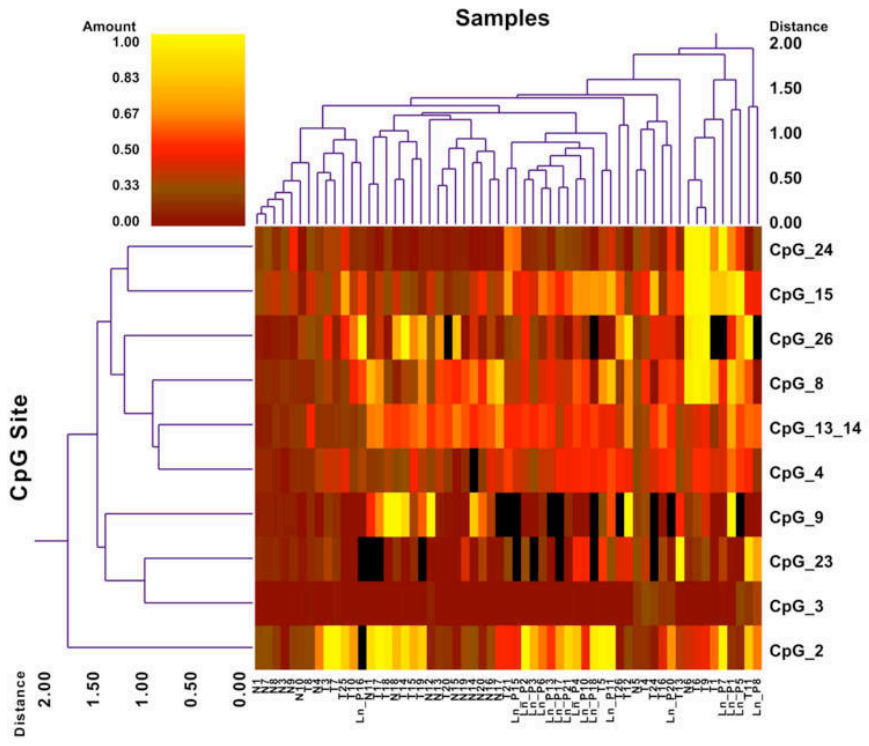
Supplementary Data

High-throughput methylation analysis of CpG sites in 12 candidate genes related to breast cancer.

APC Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
324	adenomatous polyposis coli	5q21-q22	Cell adhesion, signal transduction, stabilization of the cytoskeleton, regulation of cell cycle and apoptosis	direct

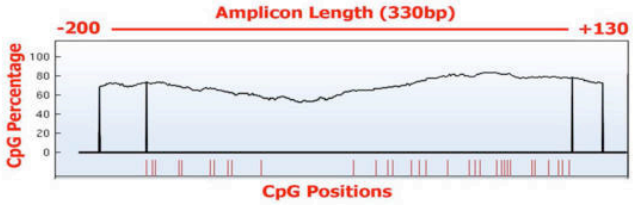


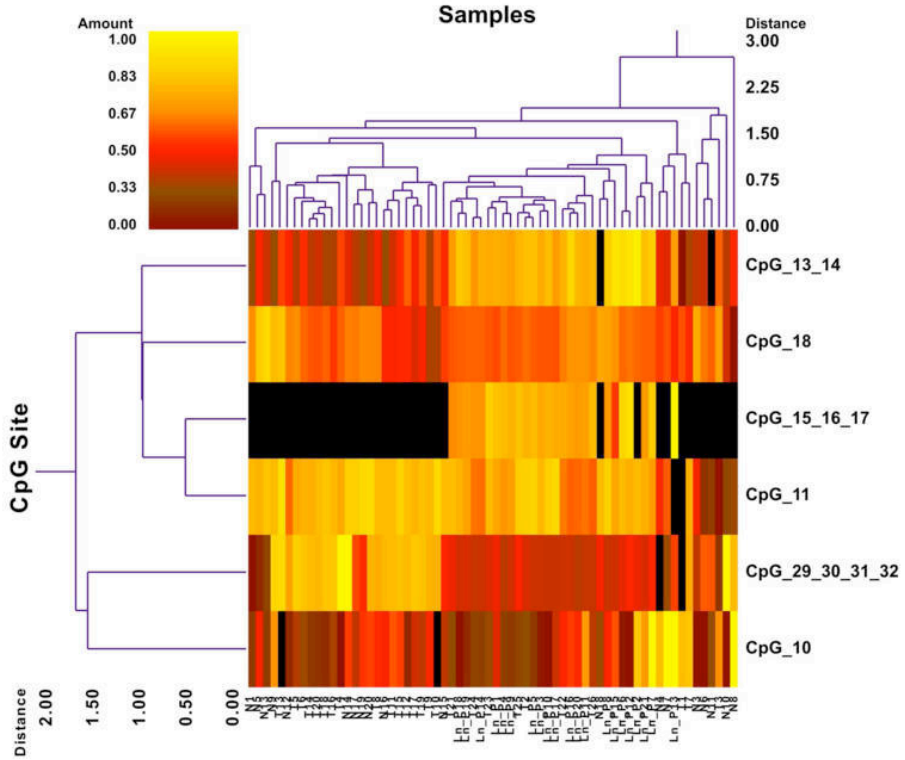


Double dendrogram of APC gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

BIN1 Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
274	Bridging integrator 1	2q14	Encodes several isoforms of a nucleocytoplasmic adaptor protein, one of which was initially identified as a MYC-interacting protein with features of a tumour suppressor.	direct

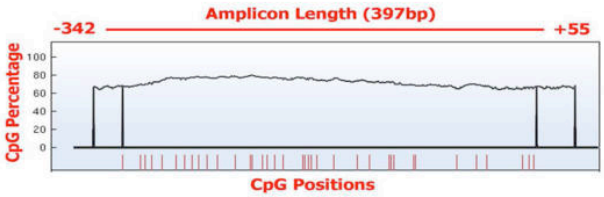


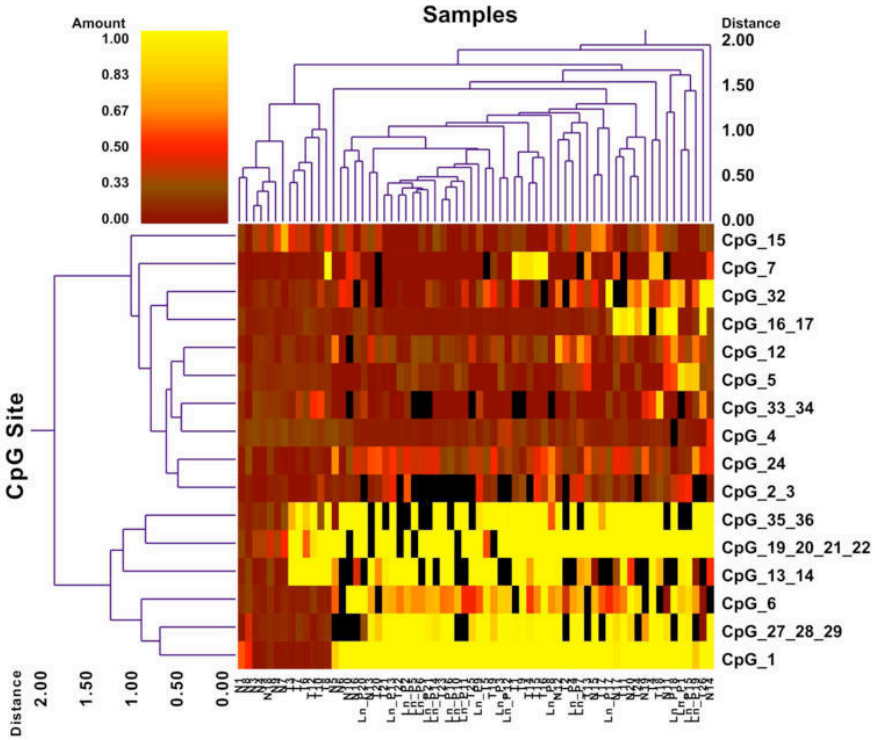


Double dendrogram of BIN1 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

BMP6 Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
654	bone morphogenetic protein 6	6p24-p23	The bone morphogenetic proteins (BMPs) are a family of secreted signalling molecules.	direct

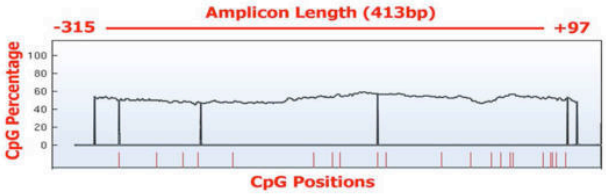


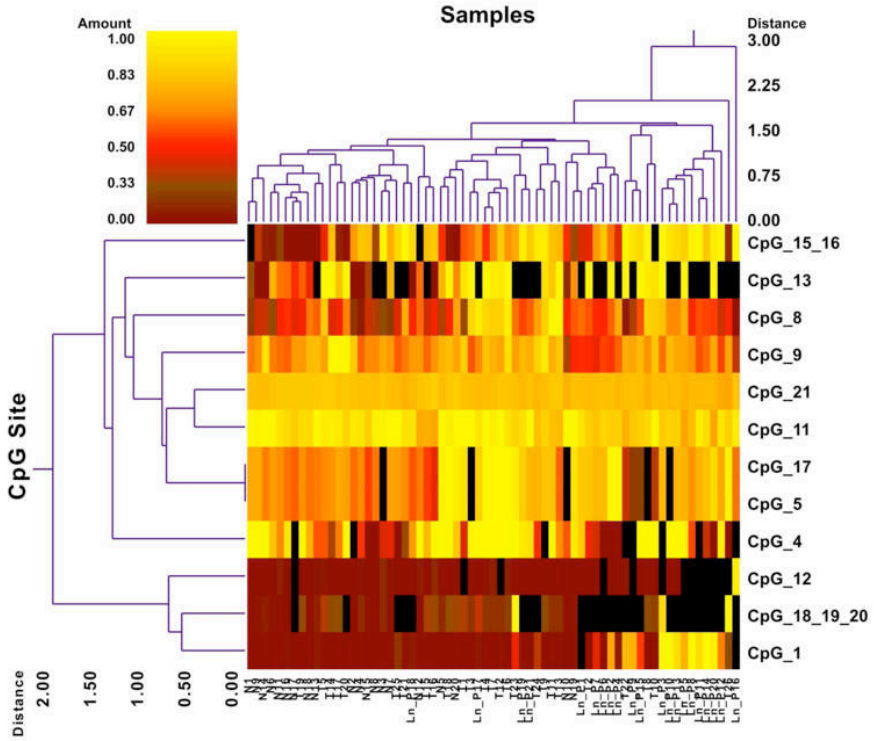


Double dendrogram of BMP6 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

BRCA1 Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
672	Breast Cancer type 1	17q21	Involved in DNA repair, recombination, checkpoint control of the cell cycle and transcription. Interacts with p53, STAT-factors, SRBC, etc.	direct

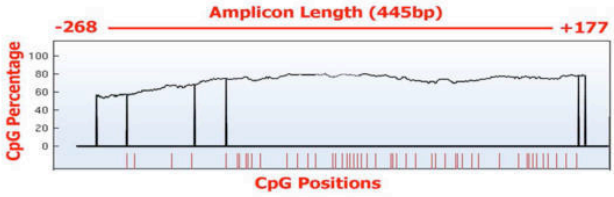


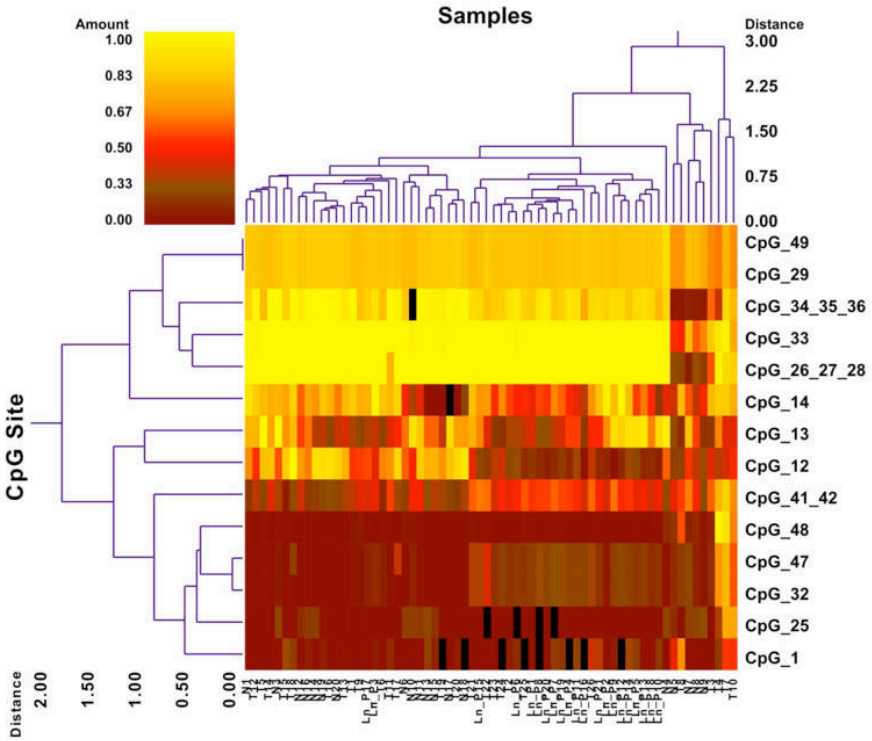


Double dendrogram of BRCA1 **gene:** Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

CST6 Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
1474	Cystatin E/M	11q13	This gene encodes a cystatin from type 2 family, which is down-regulated in metastatic breast tumour cells as compared to primary tumour cells. Loss of expression is likely associated with the progression of a primary tumour to a metastatic phenotype.	direct

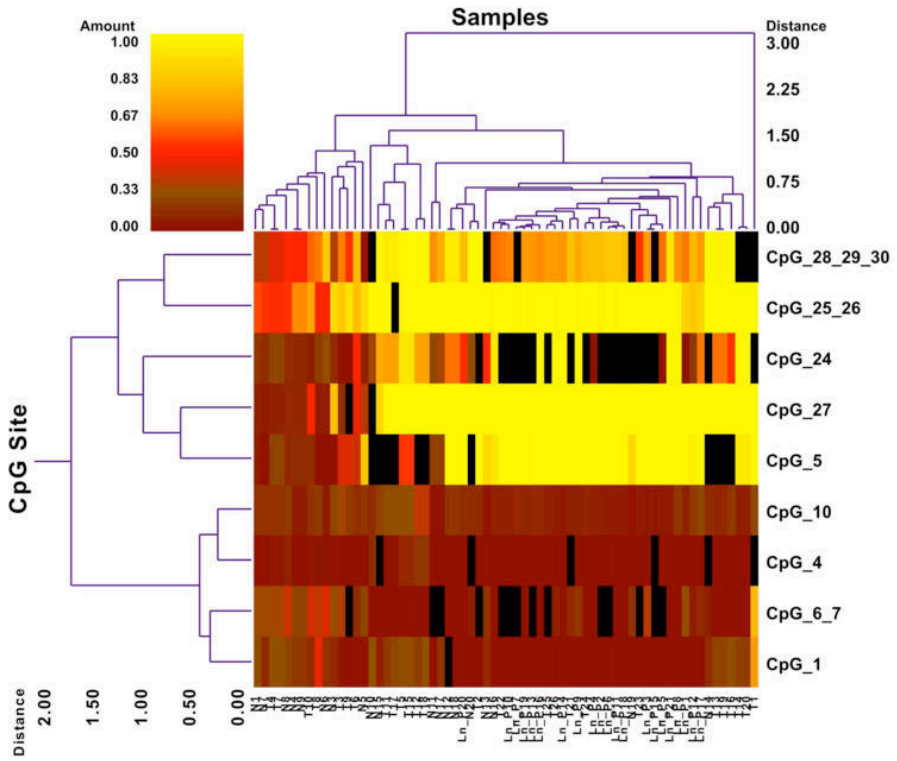




Double dendrogram of CST6 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

ESR-b Gene

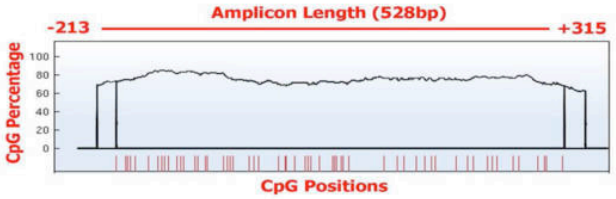
Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
2100	estrogen receptor 2 (ER beta)	14q23.2	Regulation of cell proliferation, predictor of endocrine therapy	direct

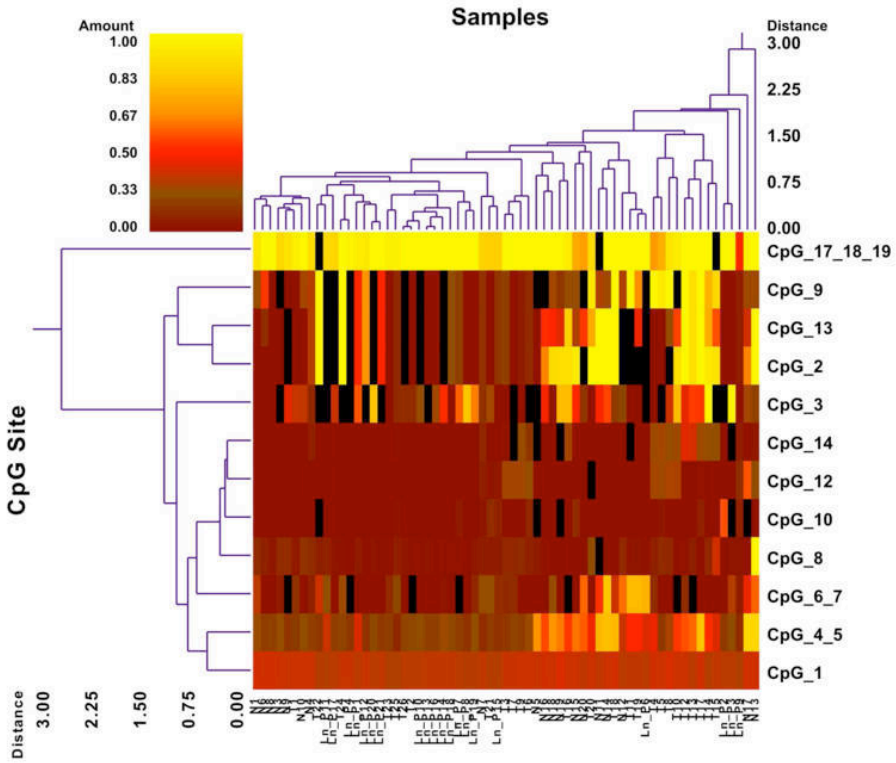


Double dendrogram of ESR-b gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

GSTP1 Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
2950	Glutathione S-transferase P1	11q13	Carcinogen detoxification. GSTP1 is a polymorphic gene encoding active, functionally different GSTP1 variant proteins that are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer, and other diseases.	direct

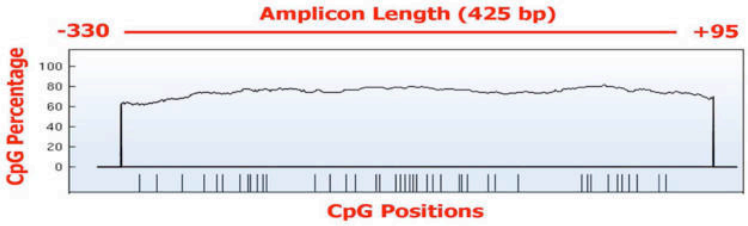


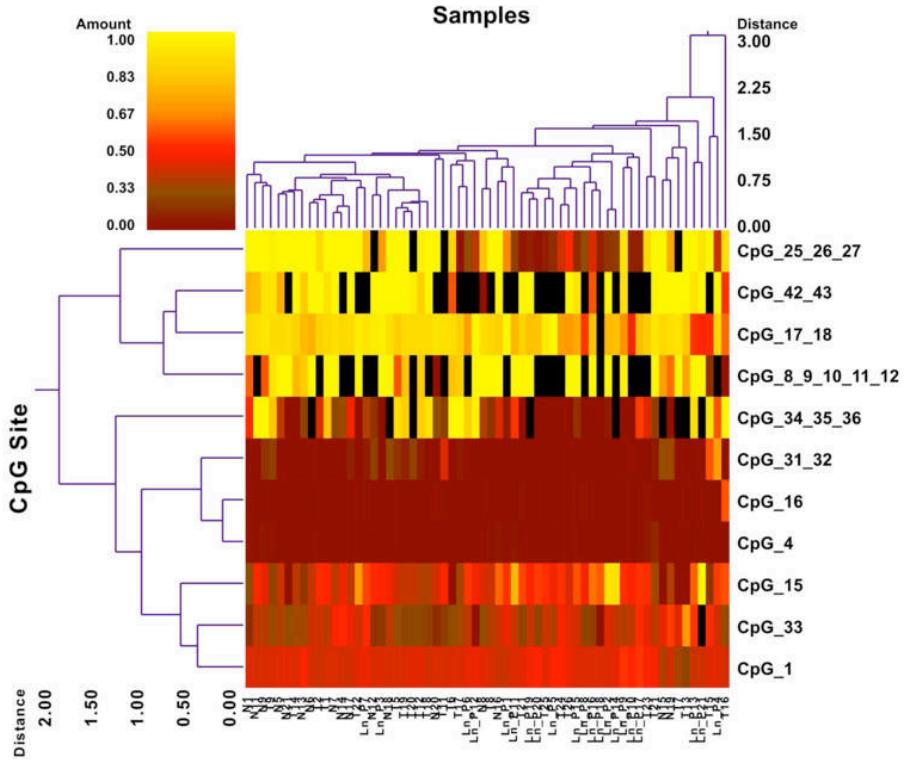


Double dendrogram of GSTP1 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

P14^{ARF} Gene

Gene ID	locus	Function	Methylation effect on breast cancer
1029	9p21	Cell cycle regulation, involved in senescenc: This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53.	direct

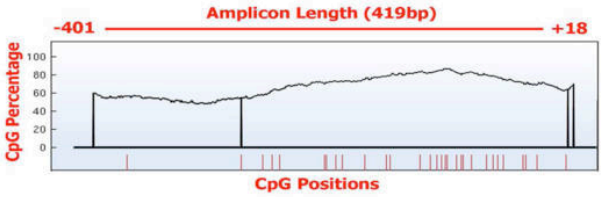


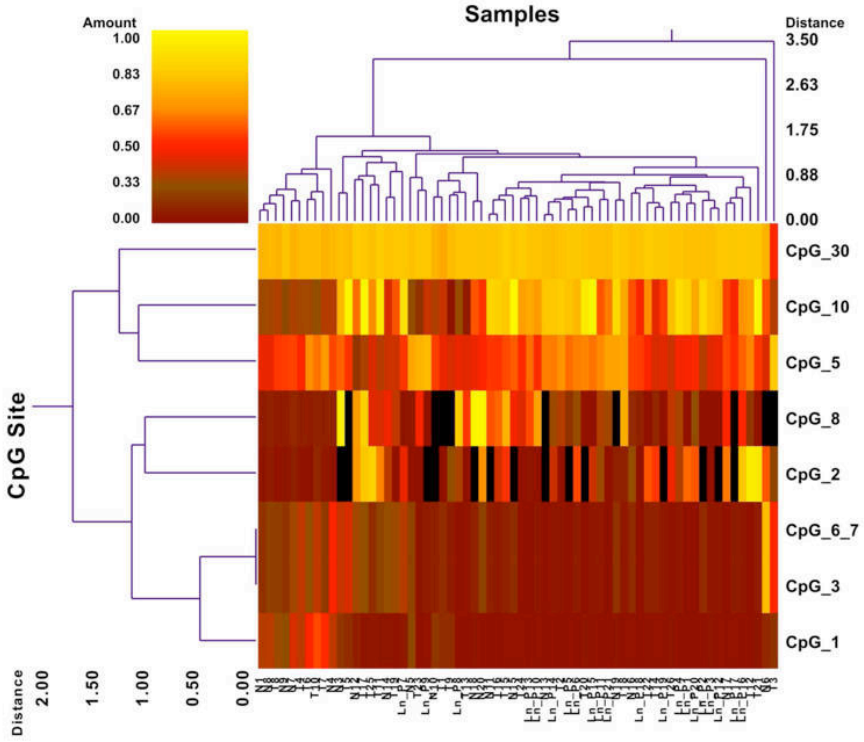


Double dendrogram of P14 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

P21 (CDKN1A) Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
1026	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	6p21.2	Encodes protein that binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. This protein plays a regulatory role in S phase DNA replication and DNA damage repair.	direct

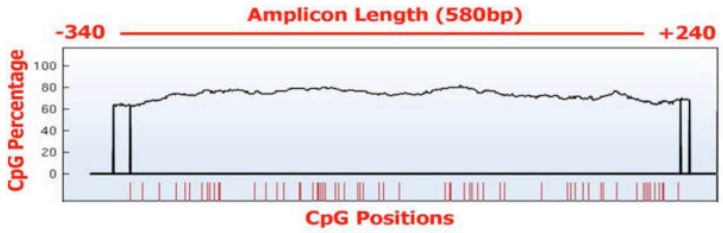


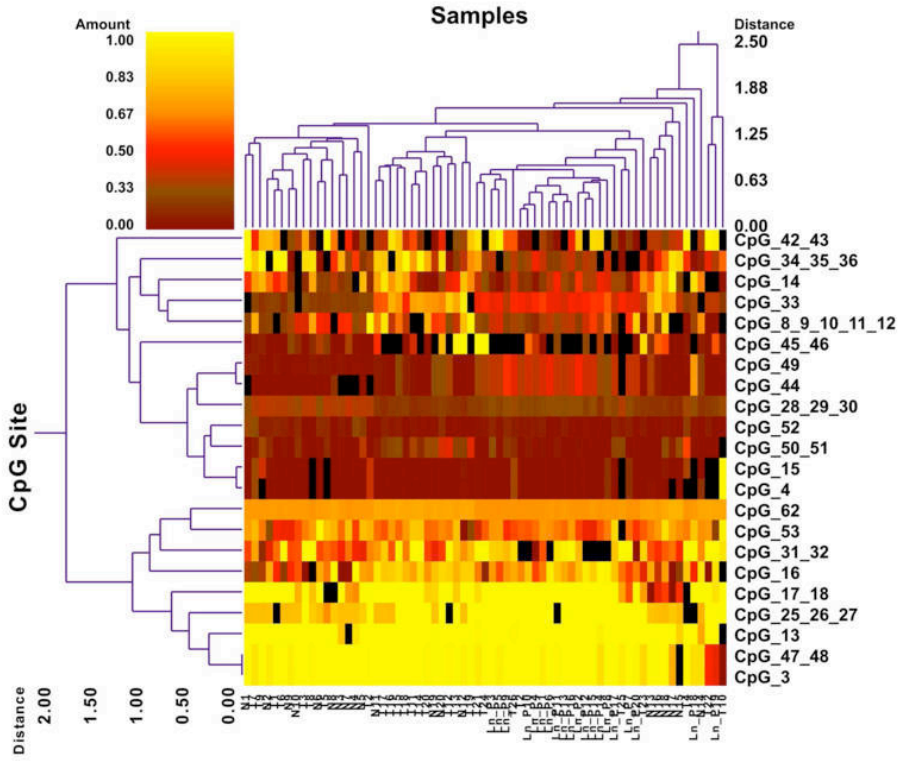


Double dendrogram of P21 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

P16 (CDKN2A) Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
1029	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	9p21	Cell cycle regulation, involved in senescence.	direct

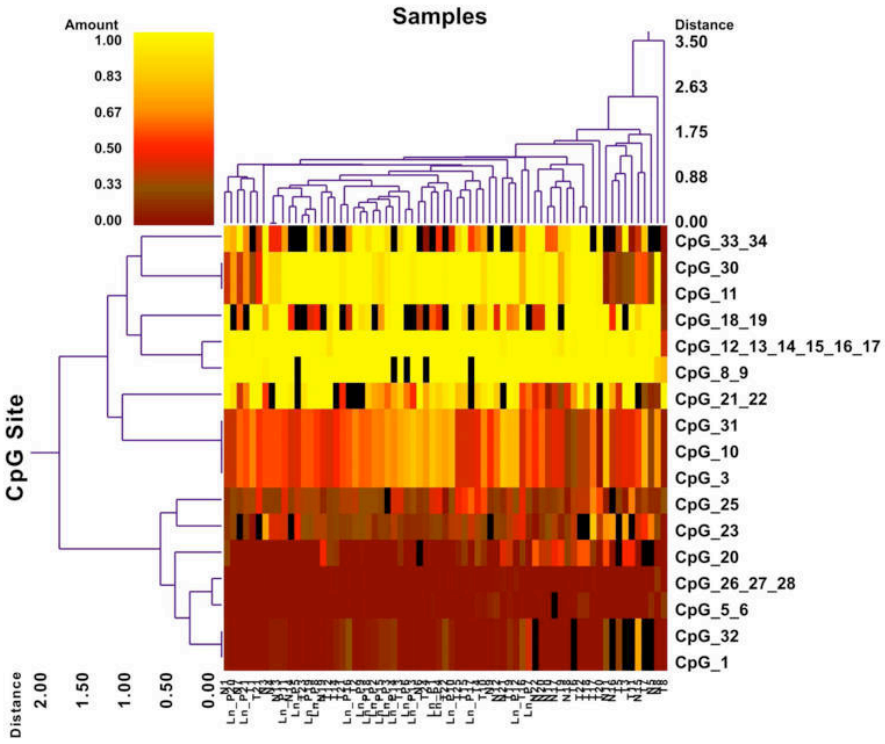




Double dendrogram of P16 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

PTEN Gene

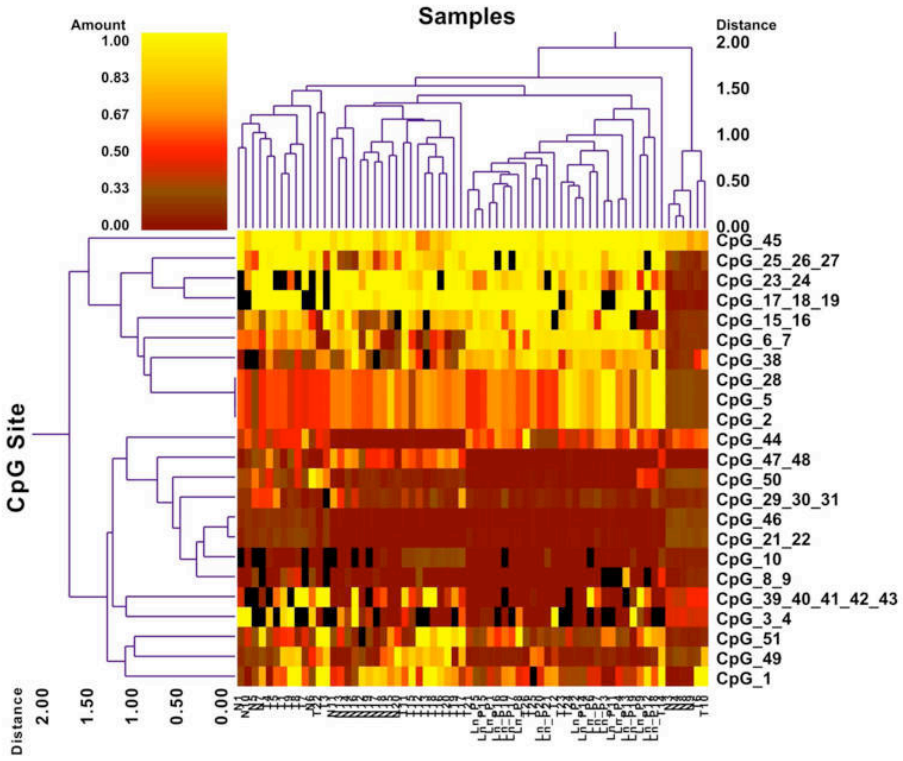
Gene ID	locus	Function	Methylation effect on breast cancer
5728	10q23.3	This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase.	direct



Double dendrogram of PTEN gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

TIMP3 Gene

Gene ID	Alternate gene name	locus	Function	Methylation effect on breast cancer
7078	Tissue inhibitor of metalloproteinase-3	22q12.3	Suppresses tumour growth, angiogenesis, invasion and metastasis	direct



Double dendrogram of TIMP3 gene: Two-way hierarchical cluster analysis of breast cancer studied cohort. (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100, and black clusters indicate not analyzed CpG sites).

3.5 Under-review manuscript

Proteomics Investigation to Identify p53-Associated Proteins Upon Demethylation Treatment in the Presence or Lack of *TP53* Mutation

Author: Zeinab Barekati, Ramin Radpour, Corina Kohler, Thomas Grussenmeyer, Paul Jenoe, Martin M. Schumacher, Suzette Moes, Frank Staedtler, Xiao Yan Zhong, Ivan Lefkovits.

Summary: The contribution of aberrant DNA hypermethylation in gene silencing and carcinogenesis has been demonstrated. DNA hypermethylation is a reversible alteration. Here, on the basis of demethylation therapy and *in-silico* pathway analysis we achieved an over-all proteomic characterization of wild type and mutant p53 breast neoplasm. Our founding might provide a further clue to establish meaningful biomarkers for breast cancer patients.

Author contributions: Zeinab Barekati was involved in experimental design, performing the study, data analysis, interoperating data and writing the manuscript.

Running title: Proteomics investigation of demethylation treatment

Proteomics Investigation to Identify p53-Associated Proteins Upon Demethylation Treatment in the Presence or Lack of *TP53* Mutation

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Abstract

The contribution of aberrant DNA hypermethylation in gene silencing and carcinogenesis has been demonstrated. Considering aberrant DNA hypermethylation as a reversible alteration, on the basis of demethylation therapy and *in-silico* pathway analysis we achieved an over-all proteomic characterization of breast neoplasm. The present proteomic investigation elucidated a possible mechanism of DNA methylation influencing expression of proteins involved in the p53 pathway and the potential link of the detected proteins to the biological processes, in particular mitochondria and telomere maintenance in wild-type (wt) *TP53* (MCF7) and mutant *TP53* (HS578T and BT549) breast cancer cell lines. Dysregulation of Atic, Calr and Pcna were characterized in the wt-p53 cell line. Furthermore, the *in-silico* pathway analysis revealed association between aberrant expression of Prdx3 and Pcna to mitochondria and telomere maintenance for the studied breast cancer cell lines. Taken together, these finding might provide a further clue to establish meaningful biomarkers for breast cancer patients.

Key words: Cancer proteomics; Breast cancer; *TP53* mutation, DNA methylation; mass spectrometry; Proteomics.

Introduction

Both tumor suppressor p53 protein and its signaling pathway have an important role in maintaining genomic stability and in preventing neoplastic development (1-4). Dysfunction of p53 has been reported in many types of cancer including breast neoplasm. Loss of p53 function can be determined by genetic or epigenetic alterations of the actual gene or by alteration of the p53 related signaling pathway which consists of hundreds of genes (5-7). The frequency of mutational inactivation of the *TP53* gene in breast cancer is approximately 20% which is lower than in other types of cancer (8). Therefore, this provides a substantial consideration for exploring epigenetic alteration of *TP53* regulatory genes in breast cancer lacking a *TP53* mutation. Recently, several studies focused on aberrant methylation patterns of upstream and/or downstream *TP53* regulatory factors that may impair the pathway (9, 10); nevertheless the actual mechanism of p53 inactivation or suppression in breast cancer is still poorly understood.

Beyond several biological responses through the p53 pathway, this pathway plays an important role in the regulation of mitochondrial function and telomere maintenance (11-14). The majority of mitochondrial proteins are nuclear gene products, thus the crosstalk between mitochondria and nuclear genes is essential for mitochondrial homeostasis and biogenesis. Recently, association of nuclear promoter hypermethylation with mtDNA alteration has been reported in cancer (15, 16). Several studies in this area (17, 18) as well as our own work (9) have indicated that mtDNA vulnerability such as mutation and mtDNA content were influenced by *TP53* mutation or promoter hypermethylation of the genes involved in p53 pathway.

Along with the influence of impaired p53 pathway on mitochondria, the integrity of this pathway is crucial to cooperate in response to telomere dysfunction and inhibition of tumorigenesis. Dysfunction of p53 (and the actual pathway) contributes to bypass the senescence checkpoints and leads to telomere dysfunction in cancer (14, 19-21). In our earlier work we have demonstrated The correlation between telomere shortening and hypermethylation of the p53 pathway in breast cancer patients (10).

The present study investigated the dysregulation of protein expression influenced by DNA methylation alteration in breast cancer cell lines with and without p53 mutation after an effective demethylation treatment with 5-aza-2′-deoxycytidine (DAC). Using LC-MS-MS analysis, proteins were identified and a comprehensive *in-silico* pathway analysis was performed to understand the bridge between the p53 pathway and epigenetic alteration with characterizing the biological responses, which allowed us to detect aberrant proteins expression involved in mitochondrial function and in telomere maintenance in breast neoplasm.

Materials and Methods

Cell culture and treatment

Three breast cancer cell lines (MCF7 (22), HS578T (23) and BT549 (24)) were treated with the effective demethylating dosage of 5-aza-2′-deoxycytidine (DAC) at a concentration of 10^2 nM as determined in a previously study.(25)

The characteristics and media conditions for each cell line are summarized in the table 1.

Mutation analysis of *TP53*

The genetic integrity of the *TP53* gene was checked by direct sequencing of exons 5-8 in each cell line as described in the previous work.(9) Direct DNA sequencing was performed by using a Big Dye terminator v3.1 cycle sequencing kit and automated sequencer was performed (ABI 3130, Applied Biosystem). The results of DNA sequence analysis were compared with the reference sequences of the gene bank (www.ncbi.nlm.mih.gov/nucleotide) using DNASTAR sequence alignment software (DNASTAR Lasergene 8, Inc., Madison, USA). All sequencing reactions were performed in both directions and confirmed for concordance.

Methylation quantification

Based on our previous study where we describe an effective DAC treatment for demethylating cancer related genes (25), we selected *CDKN1A* which is involved in the p53 pathway to confirm optimal demethylation. Promoter methylation status of the candidate gene was quantified using thymidine-specific cleavage mass array on MALDI-TOF MS according to the previously published methods (26-29).

Bisulfite treatment*;* The Epitect® Bisulfite Kit (QIAGEN AG, Basel, Switzerland) was used to perform bisulfite conversion of the target sequences.

Primer designing and PCR-tagging for EpiTYPER™ assay*;* the CpG density and CpG sites of the target sequence for *CDKN1A* was analyzed for the PCR primer design. The designed and tagged primers (reverse primer with T7-promoter tag and the forward primer with a 10mer tag sequence as balance) were applied for the experiment. The primer sequences and the PCR condition are summarized in supporting information.

In vitro transcription, T-cleavage assay and Mass spectrometry. In vitro transcription and T-cleavage were assessed according to the previously published methods (26, 27, 30), Twenty-two nanoliters of cleavage reaction were robotically dispensed (nanodispenser) onto silicon chips preloaded with matrix (SpectroCHIP; SEQUENOM, San Diego). Mass spectra were collected using a MassARRAY Compact MALDI-TOF (SEQUENOM) and spectra's methylation ratios were generated by the Epityper software v1.0 (SEQUENOM, San Diego).

Proteomic profiling

Protein pellets from AllPrep® extraction (5 x 10^6 cells per sample) were directly solubilized in a buffer consisting of 7 M urea, 2 M thiourea, 4 % CHAPS, 20 mM DTT and 2 % ampholines, pH 3-10 (Invitrogen) for a total volume of 0.1 mL.

Two-dimensional gel electrophoresis (2DE) and spot visualization. 2D electrophoresis for separation was done using ISODALT system. The first dimension was performed by isoelectric focusing (ampholines pH 3-10 (Invitrogen) (31, 32); and for the second dimension a 11-19% linear acrylamide gradient was used. Protein

spots were visualized by silver staining and wet silver-stained gels were scanned using a Pharmacia Image Scanner with 300 dpi at 16 bit, based on a published method (28). The methodology of using silver staining for quantitative evaluation of proteome has been previously established (31).

Image analysis and spot quantification. Each protein sample was subjected to 2DE in duplicate in order to evaluate gel reproducibility and to ensure reliability of measurements of the qualitative and quantitative changes in protein expression. Progenesis SameSpot software (v 4.0, NonLinear Dynamics, UK) was used for gel alignment, spot detection, spot quantification, and normalization for the total spot volume in each gel, and the data were statistically analyzed using the incorporated statistical package. Gel images were automatically aligned after manually assigning 20 landmark vectors. The ANOVA test is used here to assess the differentially expressed proteins by comparing normalized volume of protein spots. As applied in this investigation it compares always a set of four congruent spots – two from untreated and two from treated set. Prior to establishing the statistical significance ($P<0.05$) of the two treatments the values of the replicas are compared. Only if these two replica values are "close", the two treatments are evaluated for significance. Since this procedure takes in consideration four values (the weight of the evaluation is better than such of a triplicate), we refer to it as a 2x2 ANOVA.

Mass Spectrometry and Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS)

The protein spots of interest were manually excised from the gels and underwent in-gel digestion with trypsin. Samples of the trypsin digested proteins were analyzed by capillary liquid chromatography tandem MS (LC-MS-MS) using a setup of a ProteoCol trap C-18 column (0.15 x 10mm, 3 μm particle size, 300Å) (SGE Analytical Science, Victoria, AU) and a separating column (0.1mm x 10cm) that had been packed with Magic 300Å C18 reverse-phase material (5 mm particle size, Swiss Bioanalytics, Birsfelden, Switzerland). The columns were connected on line to an Orbitrap FT hybrid instrument (Thermo Finnigan, San Jose, CA, USA). The solvents used for peptide separation consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid and 80% acetonitrile in water (solvent B). Peptides were injected via a 2 μL loop onto the trap column with the

capillary pump of an Agilent 1200 system set to 5 μL/min. After 15 min, the trap column was switched into the flow path of the separating column. A linear gradient (2 – 35%) of solvent B in solvent A in 60 min was delivered with an Agilent 1200 nano pump at a flow rate of 500 nL/min. After 60 min the percentage of solvent B was increased to 60% in ten minutes and further increased to 80% in 2 min. The eluting peptides were ionized at 1.7 kV. The mass spectrometer was operated in a data-dependent fashion. The precursor scan was done in the Orbitrap set resolution of 60,000, while the fragment ions were mass analyzed in the LTQ instrument.

Peptide identification. The MS-MS spectra were matched against the human data bank (NCBI non-redundant, version October 1st, 2010) using the TurboSequest software.(33) The data bank was searched with 10 ppm precursor ion tolerance, while the fragment ions were set to a 0.5 Da tolerances. Cleavage rules were set to fully enzymatic – cleaves at both ends, allowing 2 missed cleavages. Post filtering was set to the following parameters: ΔCN, 0.1; Xcorr versus charge state was 1.50 (1+), 2.00 (2+), 2.50 (3+); peptide probability 0.01; protein probability 0.01. Valid identification required two or more peptides independently matching the same protein sequence, a significant peptide score (P<0.05), and the manual confirmation of agreement between the spectra and peptide sequence. Protein quantification and data validation was performed using Scaffold (version Scaffold 3.00.06; Proteome Software, Portland, OR, USA), which models the score distributions of the entire dataset of spectra. Database search files generated by TurboSequest were imported into Scaffold and analyzed using the tandem searches against the same protein sequence database using the same search parameters as the associated TurboSequest search.

Cell signaling and pathway analysis

Gene networks and canonical pathways representing detected proteins were identified using the Pathway Studio® software version 7.1 and ResNet® 7 (Mammal) database (Ariadne Genomics, Inc., Rockville, USA). The functional analysis identified the biological functions and/or diseases that were most relevant to the data sets and facilitated the understanding beyond their functional link to breast neoplasm. This analysis provided an approach to compare different kinds of cellular interactions, including protein-protein interactions.

Gene expression analysis

All the studied samples were subjected to simultaneous isolation of DNA, RNA and protein using AllPrep® DNA/RNA/Protein Mini Kit (QIAGEN AG, Basel, Switzerland) according to the published protocol (28). The cDNAs were synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). For quantitative Real-Time PCR (qRT-PCR) analysis, synthesized cDNAs amplified with specific gene primers using SYBR® Green 2X PCR Master Mix (Applied Biosystems, UK). The primer sequences are presented in supporting information table S3. Real-time PCR reactions were performed in two replicates and including no-template controls using ABI Prism 7500 Sequence Detection System (Applied Biosystems). At least three biological replicate were considered for each treatment group. The fold difference for each sample was calculated using Livak method and relative to un-treated cell lines (34).

Results and discussion

TP53 mutation screening

The major *TP53* mutation has been found in the DNA binding domain (exon 5-8) which has a crucial role for the biological activity of the gene.(35) The results of direct sequencing of *TP53* exon 5-8, for the breast cancer cell lines were analyzed and compared with the P53 GenBank sequence (NC_000017 [gi:224589808]). The Exon 5 mutation screening in this study showed a transversion mutation (c.12350 G<T) in both BT549 and HS578T cell lines. For HS578T cell line a transition mutation (c.13713 G<A) was found in exon 8. The MCF7 cell line showed no mutation for these analyzed exons (dataset S1; Fig.1).

Quantitative methylation profiling

The *CDKN1A* gene is involved in the p53 pathway and it was selected to confirm the effective dosage of DAC for DNA demethylation treatment. For *CDKN1A* gene, one amplicon containing a CpG rich-island was analyzed. The mean methylation quantity of the informative CpG sites for this gene was used to assess the

methylation proportion of the candidate gene. DNA methylation proportion of the *CDKN1A* was significantly decreased after DAC treatment ($P<0.05$) (Fig 2, S1).

Protein expression profiling

A proteome-based approach was performed to assess the effect of DNA methylation of the genes that are associated with P53 pathway, mitochondrial function and telomere maintenance in breast cancer cell lines. Proteins from the three breast cancer cell lines (MCF7, HS578T and BT549) were extracted after treatment with an optimal dosage of DAC (25) and were analyzed by 2D gel electrophoresis. The protein expressions were compared before and after treatment for all experimental cell lines.

For MCF7, a total of 12 differentially expressed protein spots were detected and HS578T breast cancer cell line revealed 10 differentially expressed protein spots. Interestingly, the BT549 cell line had the highest number of differentially expressed protein spots with the total of 23. The gel replicates and their significantly altered protein spot pattern for each cell line are illustrated in Figure 3.

The differentially expressed protein spots were excised and subjected to LC-MS-MS analysis. In some excised protein spots we identified a single protein and in others several proteins were observed. In all instances where the probability of correct determination was close to 100% (as determined by Scaffold) we have checked the observed molecular mass and P value (according to spot position on the gel) with the MS identification. The spot volumes, fold change and P value are summarized in Table 2.

From the differentially expressed protein species, twenty two proteins from MCF7, eleven proteins from HS578T and twenty two proteins from BT549 were eventually identified by LC-MS-MS. Among the identified proteins, some proteins showed similar expression trends in response to the demethylation treatment such as over-expression of 60 kDa heat shock protein, mitochondrial (Hspd1), Copine-3 (Cpne3) and Zinc-alpha-2-glycoprotein precursor (Azgp1) in HS578T and BT549 cell lines. Some other proteins showed different expression trends in a cell line dependent manner such as Thioredoxin-dependent peroxide reductase (Prdx3)

up-regulation in BT549 and down-regulation in MCF7 and Caspase-14 precursor (Casp14) up-regulation in HS578T and down-regulation in MCF7. The complete list of detected proteins using capillary liquid chromatography tandem MS within three analyzed cell lines are presented in Table 3.

Pathway analysis

To obtain a global view about the identified proteins, the molecular function, biological processes, association to p53 pathway, mitochondrial function and telomere maintenance were analyzed by ResNet® 7 (Mammal) and Swiss-Prot databases. The pathway analysis revealed a number of proteins within each studied cell line which are linked to p53 and also two intersections (the common up/down-regulated proteins in different cell lines were considered as "intersection") proteins contributed in mitochondria homeostasis and telomere maintenance upon demethylation treatment.

For the MCF7 cell line (as wt-p53 subtype) three up-regulated proteins were determined, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (Atic), calreticulin precursor (Calr) and proliferating cell nuclear antigen (Pcna) which are linked to p53 (Fig. 4). The mRNA expression of *ATIC*, *CALR* and *PCNA* were also assessed in the three studied cell lines, which showed significant up-regulation in MCF7 after demethylation treatment (fold change: *ATIC*; 3.79, *CALR*; 2.37, *PCNA;* 3.91, $P< 0.05$, Fig.5). The low expression of Atic, Calr and Pcna before DAC treatment might be explained by DNA methylation alteration that contributed to transcriptional silencing and eventually to down-regulation of protein expression.

Atic protein is a bifunctional enzyme with a catalytic role in the *de novo* purine biosynthesis (36). The pathway analysis showed a positive regulatory effect of this protein on p53. It can increase p53 expression and phosphorylate p53 that leads to regulate cell growth and induction of apoptosis (37), (38). Calr protein (CRT), a ubiquitous protein, is located in the endoplasmic reticulum and in the nuclear envelope lumen. This protein is

involved in several cellular and molecular processes such as chaperon activity for regulation of Ca^{2+} homeostasis and regulation of nuclear transport.(39, 40) The p53 function can be regulated by several mechanisms such as nuclear localization and posttranslational modifications. It has been demonstrated that Calr function is required for regulation and nuclear localization of the p53 protein and it plays a role in inhibition of cancer metastasis (41, 42).

The Pcna protein is well known in both DNA replication and DNA repair mechanism (43, 44). The Pcna protein interacts with many other proteins and has an important role in diverse cellular responses for instance in cell cycle control. Among proteins involved in cell cycle control, p53 and Pcna have reciprocal effects on each other; p53 regulates the expression level of the Pcna, while Pcna contributes to p53 stabilization[42, 43]. The differential expression of Pcna in cell proliferation and cell transformation has been reported (45-47). Furthermore, pathway analysis revealed an association between Pcna and telomere maintenance. The Pcna has been suggested as a component of the telomerase complexes that might regulate telomere synthesis(48). Deregulation of these genes suggests a possible mechanism for p53 dysfunction as well as impaired telomere maintenance in breast cancer lacking *TP53* mutations. Further in depth investigation, e.g. using MCF-7-p53 defective (p53 knock down/ out) cells, will be needed to confirm the relevance of our findings.

The only dysregulated protein in BT549 cell line linked to p53 was 26S proteasome non-ATPase regulatory subunit 10 (Psmd10). Psmd10 showed up-regulation in the BT549 cell line after demethylation treatment with DAC (Fig. 6). This protein interacts with the MDM2 gene and displaces *P14ARF* resulting in increasing ubiquitylation and degradation of p53 that eventually leads to genomic instability and oncogenic transformation (49, 50). In the HS578T cell line, we could detect an increase of the 78 kDa glucose-regulated protein precursor (Hspa5) which is involved in regulation of p53 (51). The Hspa5 protein inhibits p53-dependent apoptosis and contributes to the regulation of tumor invasion and metastasis (Fig.7) (52, 53). Since BT548 and HS578T are p53 mutant cell lines, dysregulation of Psmd10 and Hspa5 proteins may not interfere with p53 malfunction as a tumor suppressor factor.

The proteomic and pathway analysis revealed an intersection protein, thioredoxin-dependent peroxide reductase (Prdx3), involved in mitochondrial maintenance. The Prdx3 protein, a target of C-myc, was detected as an intersection protein within both BT549 and MCF7 cell lines (Fig. 4a and 6a). Prdx3 protein was up-regulated in BT549 cell line and down-regulated in MCF7 cell line after DAC treatment. In the contrary to low protein expression of Prdx3 in MCF7, the mRNA expression level was significantly up-regulated ($P<0.05$) after treatment that might be due to post-transcriptional or post-translational modification leading to repression of this gene at the level of proteome. Prdx3 protein is required for the maintenance of mitochondrial mass and function (54). Overexpression of Prdx3 protein protects the cells against apoptosis induced by hypoxia condition and it has been reported in some human cancers resulting in protection of tumor growth (55, 56). The inverse expression of Prdx3 protein in these two cell lines suggests the investigation of the p53/C-myc pathway that might be influenced by the p53 status and results in differential regulation of *PRDX3* genes.

Conclusion

Using proteomics technology, we investigated the effect of DNA demethylation treatment on the proteome of three breast cancer cell lines (MCF7, HS578T and BT549). The possible DNA methylation role in aberrant protein expression linked to the p53 pathway in the presence or lack of *TP53* mutation was studied and the known function and interaction of the detected proteins in particular relation to mitochondria and telomere maintenance were also analyzed. The present investigation suggests that DNA methylation alteration is an indirect mechanism influencing p53 by inactivation of Atic, Calr and Pcna proteins in p53 wild type breast cancer cell line. Furthermore, dysregulation of Prdx3 and Pcna have been shown by *in silico* pathway analysis to be linked to mitochondria and telomere maintenance. These proteins might provide a further clue to establish meaningful biomarkers for breast cancer patients.

Supporting information

The sequence of PCR tagged primers for *in vitro* transcription and also primers for *TP53* gene sequencing is

available in Dataset S1.

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Table 1. Source, clinical and pathological features, and culture conditions of used breast cell lines.

Cell line	TP53	Source	Tumor Type	Culture media
BT549	++M	P.Br	IDC, pap	RPMI
HS578T	++M	P.Br	IDC	DMEM
MCF7	+/+WT	PE	IDC	DMEM

P.Br, primary breast; PE, pleural effusion. TP53 status: TP53 mutational status obtained from exon 5-8 analysis(M, mutant; WT, wild-type protein). Media conditions: FBS, fetal bovine serum (10%); cultured at 37°C and 5% CO_2; DMEM, Dulbecco's modified Eagle's medium, GIBCO #11965-092; RPMI, RPMI medium 1640, GIBCO #27016-021.

Table 2. Differentially expressed proteins in breast cancer subtypes found by 2D gels.

	Spot No.	Average Normalized Volumes		Fold change	Anova (p)
		Un-treated ($\times 10^6$)	Treated ($\times 10^6$)		
BT549	1869	1.416	9.379	- 1.5	0.005
	1457	2.831	2.348	- 1.2	0.007
	1076	4.740	2.334	- 2.0	0.009
	2503	9.415	14.09	+ 1.5	0.011
	1872	9.201	6.624	- 1.4	0.012
	2460	14.92	12.37	- 1.2	0.013
	849	8.008	9.515	+ 1.2	0.014
	2078	4.322	7.645	+ 1.8	0.016
	1640	12.37	15.91	+ 1.3	0.017
	1331	4.801	5.635	+ 1.2	0.019
	1898	20.14	16.24	- 1.2	0.020
	2306	4.471	4.031	- 1.1	0.024
	2049	1.852	2.464	+ 1.3	0.027
	786	15.86	19.73	+ 1.2	0.027
	1241	6.090	4.893	- 1.2	0.028
	1977	19.66	26.27	+ 1.3	0.028
	2780	3.526	7.209	+ 2.0	0.028
	1550	3.330	2.140	- 1.6	0.033
	1212	5.629	3.045	- 1.8	0.040
	1905	18.73	24.16	+ 1.3	0.046
	2635	20.40	26.80	+ 1.3	0.047
	1953	6.905	10.49	+ 1.5	0.047
	2673	6.214	4.952	- 1.3	0.050
HS578T	643	3.001	4.874	+ 1.6	0.002
	1959	5.940	6.584	+ 1.1	0.003
	1894	1.065	6.187	- 1.7	0.006
	1709	3.003	2.510	- 1.2	0.016
	1494	1.072	7.462	- 1.4	0.019
	549	5.930	6.553	+ 1.1	0.023

	Spot No.	Average Normalized Volumes		Fold change	Anova (p)
		Un-treated ($\times 10^6$)	Treated ($\times 10^6$)		
	1448	2.317	1.862	- 1.2	0.028
	1674	1.029	1.320	+ 1.3	0.030
	1908	4.826	2.862	- 1.7	0.031
	781	11.76	19.31	+ 1.6	0.036
MCF7	1531	3.695	2.904	- 1.3	0.001
	1147	10.92	9.066	- 1.2	0.007
	1373	2.312	1.486	- 1.6	0.011
	934	10.41	12.62	+ 1.2	0.011
	2314	2.030	1.430	- 1.4	0.014
	1123	4.992	5.622	+ 1.1	0.022
	1967	5.829	4.680	- 1.2	0.026
	1569	1.747	1.471	- 1.2	0.027
	1556	3.252	4.110	+ 1.3	0.036
	1063	10.98	12.74	+ 1.2	0.038
	1628	3.955	5.316	+ 1.3	0.040
	1571	1.452	1.637	+ 1.1	0.044

The spot numbering is independent in the analyzed cell lines.

(+) Up-regulated; (-) Down-regulated

Table 3. Detected proteins using LC-MS- MS within analyzed cell lines.

Cell line	No. of proteins / spot	Spot no.	Detected proteins	Gene name	Gene ID	Accession No.	MW (kDa)	No. of detected peptides	aa coverage (%)
BT549	One	1872	ADP-sugar pyrophosphatase	*NUDT5*	11164	gi|37594464	24	6	31
	One	2673	proteasome subunit beta type-9 proprotein	*PSMB9*	5698	gi|4506205	23	2	10
	One	2460	cofilin-1	*CFL1*	1072	gi|5031635	19	2	8
	One	2635 *	hypothetical protein LOC56005 precursor	*C19orf10*	56005	gi|33457348|	19	6	37
	three	1977	catechol O-methyltransferase isoform MB-COMT [	*COMT*	1312	gi|4502969	30	6	2
			proteasome subunit alpha type-3 isoform 1	*PSMA3*	5684	gi|4506183	28	55	2
			Ran-specific GTPase-activating protein - human	*RANBP1*	5902	gi|542991	23		2
	Four	1905	phosphoglycerate mutase **	*PGAM1*	5223	gi|4505753	29	4	19
				PGAM4	441531	gi|71274132	29	3	15
				PGAM2	5224	gi|51094513	28	3	10
			splicing factor, arginine/serine-rich 9	*SRSF9*	8683	gi|4506903	26	4	16
			BBC1	*RPL13*	6137	gi|29383	24	2	4
			GTP-binding nuclear protein Ran	*RAN*	5901	gi|5453555	24	2	10
	Four	2078	26S proteasome non-ATPase regulatory subunit 10	*PSMD10*	5716	gi|4506217	24	4	21
			thioredoxin-dependent peroxide reductase,	*PRDX3*	10935	gi|5802974	28	3	17
			rho-related GTP-binding protein RhoC precursor	*RHOC*	389	gi|28395033	22	2	10
			GTP-binding protein SAR1b	*SAR1B*	51128	gi|7705827	22	2	12
	Five	849	60 kDa heat shock protein, mitochondrial	*HSPD1*	3329	gi|31542947	61	37	52
			ubiquitin carboxyl-terminal hydrolase 14 isoform a	*USP14*	9097	gi|4827050	56	5	9
			copine-3	*CPNE3*	8895	gi|4503015	60	3	4
			zinc-alpha-2-glycoprotein precursor	*AZGP1*	442606	gi|4502337	34	2	3
			prolactin-inducible protein precursor	*PIP*	5304	gi|4505821	17	2	8
HS578T	One	1449	eukaryotic translation elongation factor 1 beta 2	*EEF1B2*	55949	gi|51094833	25	3	22
	Two	549	78 kDa glucose-regulated protein precursor	*HSPA5*	3309	gi|16507237	72	41	48
			annexin A6 isoform 1	*ANXA6*	309	gi|71773329	76	13	20
	Two	781 *	60 kDa heat shock protein, mitochondrial	*HSPD1*	3329	gi|31542947	61	26	49
			copine-3	*CPNE3*	8895	gi|4503015	60	2	4
	Six	643	T-complex protein 1 subunit epsilon	*CCT5*	22948	gi|24307939	60	11	18
			serpin B12	*SERPINB12*	89777	gi|17998551	46	2	2
			zinc-alpha-2-glycoprotein precursor	*AZGP1*	442606	gi|4502337	34	2	3
			annexin A2 isoform 2	*ANXA2*	302	gi|4757756	39	2	6
			caspase-14 precursor	*CASP14*	23581	gi|6912286	28	2	9
			copine-1 isoform a	*CPNE1*	8904	gi|23397696	59	2	4
MCF7	One	1063	calreticulin precursor	*CALR*	811	gi|4757900	48	11	3
	Two	1628	HCLS1-associated protein X-1 isoform a	*HAX1*	10456	gi|13435356	32	10	41
			proliferating cell nuclear antigen	*PCNA*	5111	gi|4505641	29	3	11
	Three	2314	protein mago nashi homolog 2	*MAGOHB*	55110	gi|8922331	17	5	39
			caspase-14 precursor	*CASP14*	23581	gi|6912286	28	2	9
			NADH dehydrogenase	*NDUFV2*	4729	gi|41327783	14	2	17

Five	1967	von Hippel-Lindau binding protein	*VHL*	7428	gi|2738244	19	6	27
		neighbor of COX4 isoform 1	*COX4NB*	10328	gi|5174615	24	4	24
		thioredoxin-dependent peroxide reductase, mitochondrial	*PRDX3*	10935	gi|5802974	28	4	17
		BAG family molecular chaperone regulator 2	*BAG2*	9532	gi|4757834	24	4	17
		proteasome subunit beta type-3	*PSMB3*	5691	gi|22538465	23	2	10
Eleven	1123	T-complex protein 1 subunit gamma isoform a	*TCP1*	6950	gi|63162572	61	27	41
		archain	*ARCN1ATIC*	372	gi|773575	53	17	35
		5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	*DPYSL2*	471	gi|20127454	65	12	26
		dihydropyrimidinase-related protein 2	*PAK2*	1808	gi|4503377	62	10	27
		hPAK65	*STIP1*	5062	gi|984305	55	6	15
		stress-induced-phosphoprotein 1	*CARM1*	10963	gi|5803181	63	4	9
		Histone-arginine methyltransferase CARM1	*PGM1*	10498	gi|57012689	63	3	5
		phosphoglucomutase-1 isoform 1	*CBS*	5236	gi|21361621	61	3	7
		A Unique Pyridoxal 5'-Phosphate Dependent Hemeprotein	*ARCN*	875	gi|15825696	47	2	4
		Coatomer subunit delta isoform 1	*TUBA1C*	372	gi|11863154	57	2	2
		Tubulin alpha-1C chain		84790	gi|14389309	50	2	4

* The subunits were detected in both replicates (replicate 1 & 2). There were some minor protein species which were additionally detected in each replicate and it is not presented here.

** Three different isoforms were detected for phosphoglycerate mutase protein (spot number 1905).

Figure legend

Fig. 1. Sequencing results of *TP53* gene and quantitative Promoter methylation profiling of *P21* gene in breast cancer cell lines. a1) Detected mutation in exon 5 for HS578T and Bt549. a2) Detected mutation in exon 8 for HS578T.

Fig. 2. Comparison between quantitative analysis of *P21* methylation pattern before and after DAC treatment in the studied cell lines based on the informative CpG sites.

Fig. 3. Proteomics analysis of three studied cell lines. a) Replicating of the gels. b) Spot picking gels performed from up/down-regulated proteins. c) Differential spot quantification studied for pathway analysis.

Fig. 4. MCF7 cell line. a) Protein expression and found polypeptide by LC-MS-MS. b) Pathway analysis of the identified proteins with their biological processes and association to breast carcinogenesis, neoplasm and p53.

Fig.5. The mRNA fold change differences of the three studied cell lines after demethylation treatment (*, $P<0.05$, **; $P<0.01$).

Fig. 6. BT549 cell line. a) Protein expression and found polypeptide by LC-MS-MS. b) Pathway analysis of the identified proteins with their biological processes and association to breast carcinogenesis, neoplasm and p53.

Fig. 7. HS578T cell line. a) Protein expression and found polypeptide by LC-MS-MS. b) Pathway analysis of the identified proteins with their biological processes and association to breast carcinogenesis, neoplasm and p53.

Figure.1

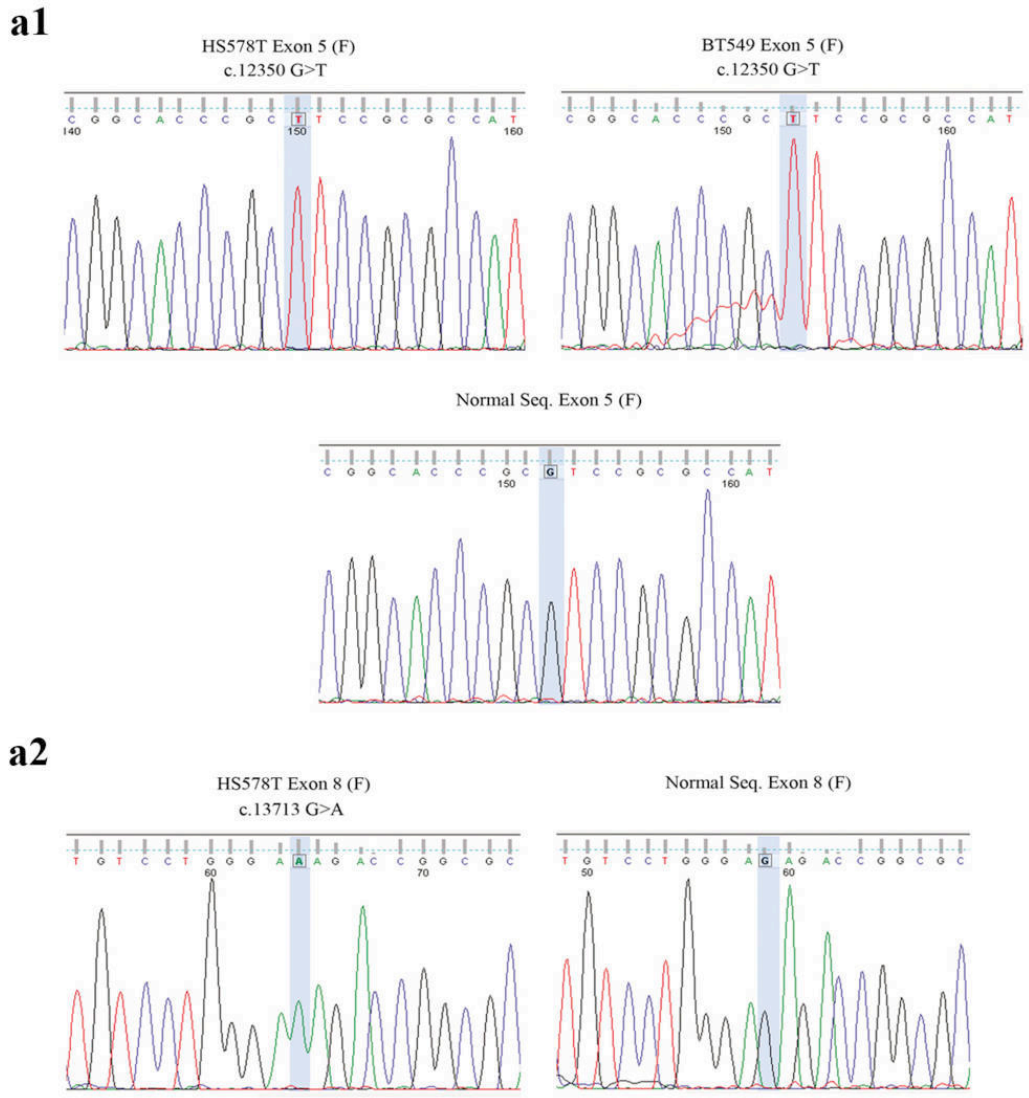

a1
HS578T Exon 5 (F)
c.12350 G>T
BT549 Exon 5 (F)
c.12350 G>T
Normal Seq. Exon 5 (F)
a2
HS578T Exon 8 (F)
c.13713 G>A
Normal Seq. Exon 8 (F)

Figure 2.

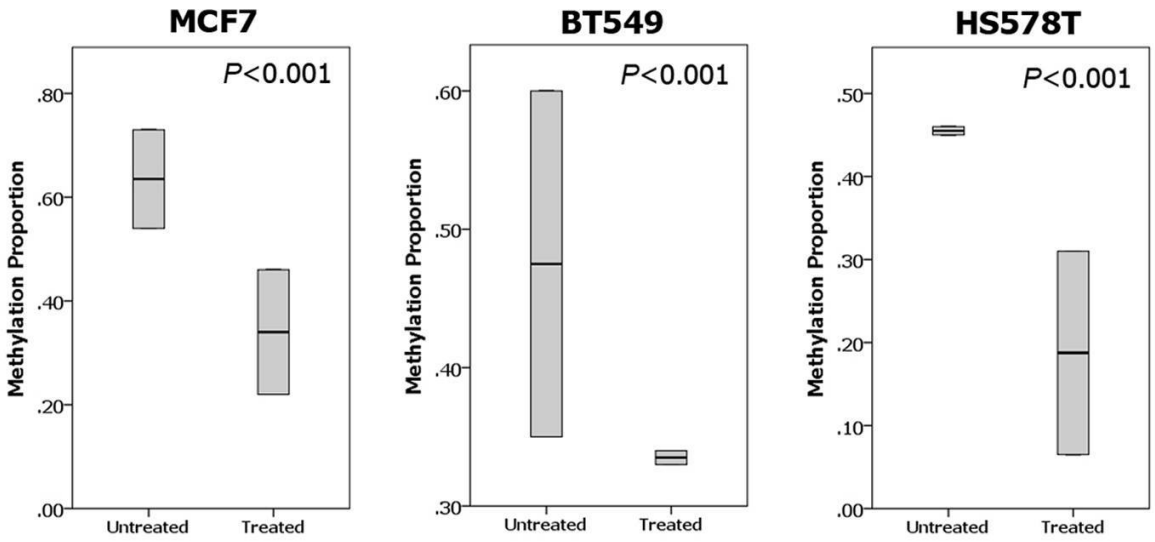

MCF7
P<0.001
Methylation Proportion
.80
.60
.40
.20
.00
Untreated
Treated
BT549
P<0.001
Methylation Proportion
.60
.50
.40
.30
Untreated
Treated
HS578T
P<0.001
Methylation Proportion
.50
.40
.30
.20
.10
.00
Untreated
Treated

Figure 3.

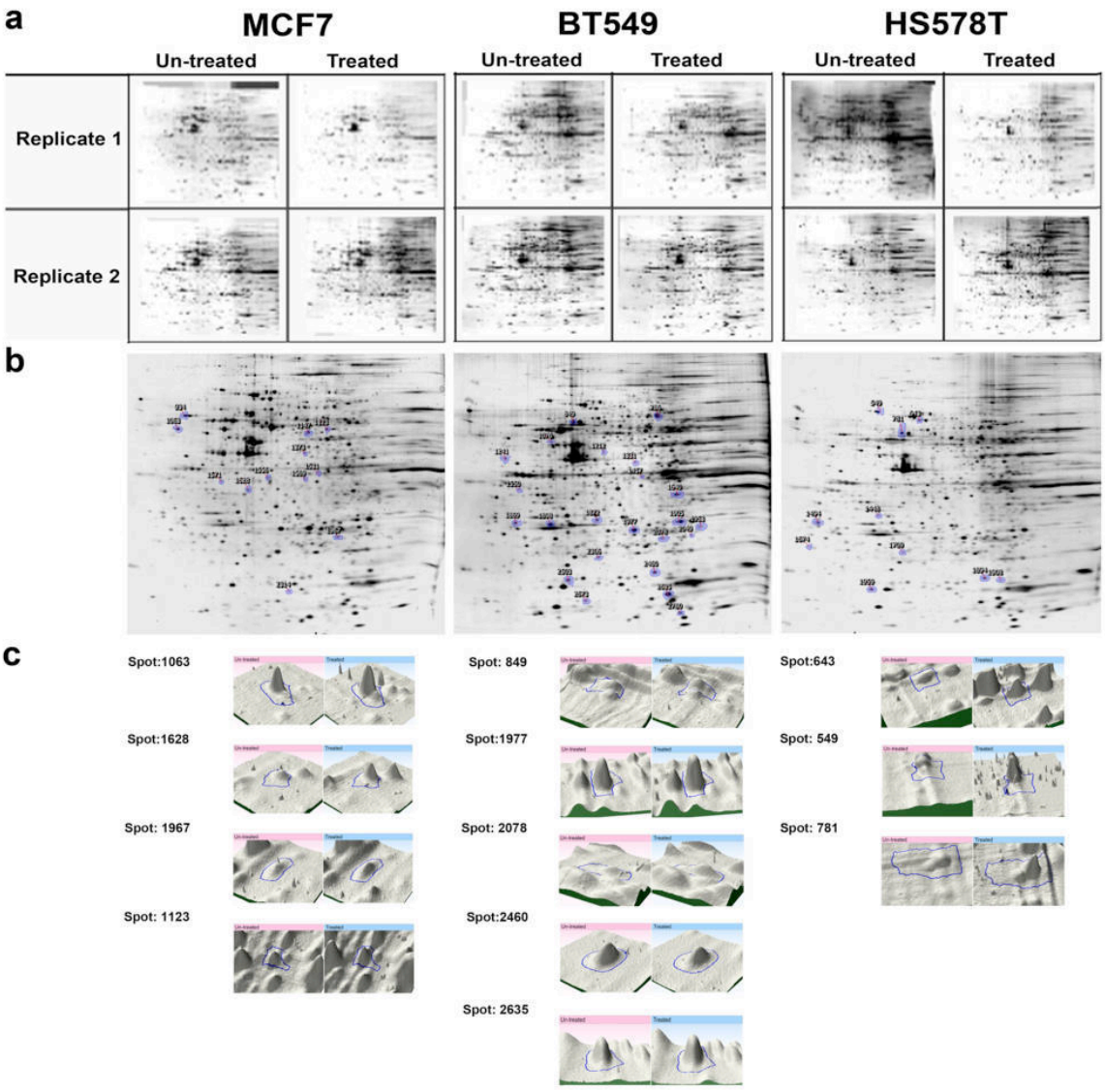

a
MCF7
BT549
HS578T
Un-treated
Treated
Un-treated
Treated
Un-treated
Treated
Replicate 1
Replicate 2
b
c
Spot:1063
Spot:1628
Spot: 1967
Spot: 1123
Spot: 849
Spot:1977
Spot: 2078
Spot:2460
Spot: 2635
Spot:643
Spot: 549
Spot: 781

Figure 4.

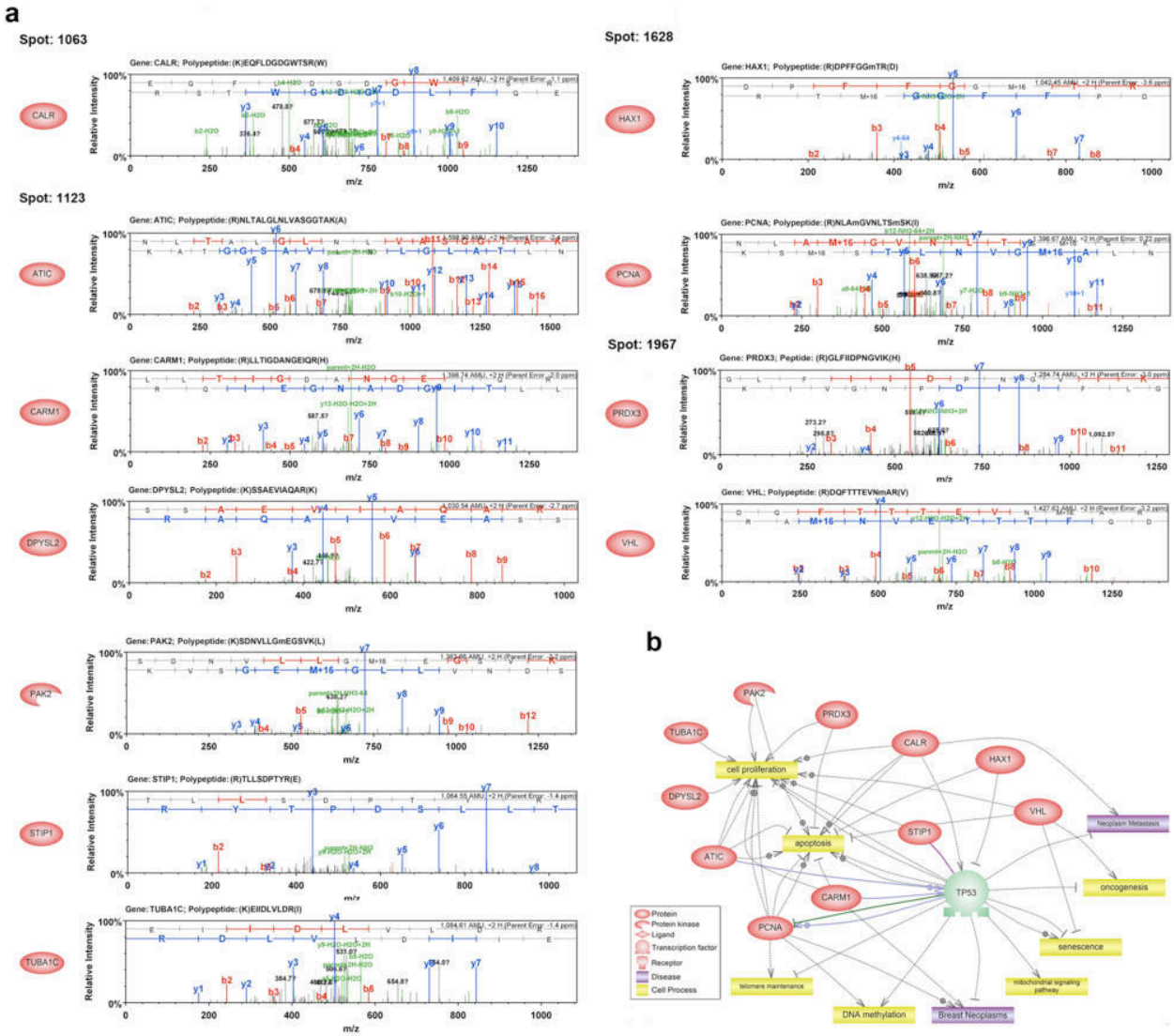

a
Spot: 1063
CALR
Spot: 1628
HAX1
Spot: 1123
ATIC
PCNA
Spot: 1967
CARM1
PRDX3
DPYSL2
VHL
PAK2
STIP1
TUBA1C
Relative Intensity
m/z
b
PAK2
PRDX3
TUBA1C
CALR
HAX1
cell proliferation
DPYSL2
VHL
Neoplasm Metastasis
STIP1
ATIC
apoptosis
TP53
oncogenesis
CARM1
PCNA
senescence
telomere maintenance
mitochondrial signaling pathway
DNA methylation
Breast Neoplasms
Protein
Protein kinase
Ligand
Transcription factor
Receptor
Disease
Cell Process

Figure 5.

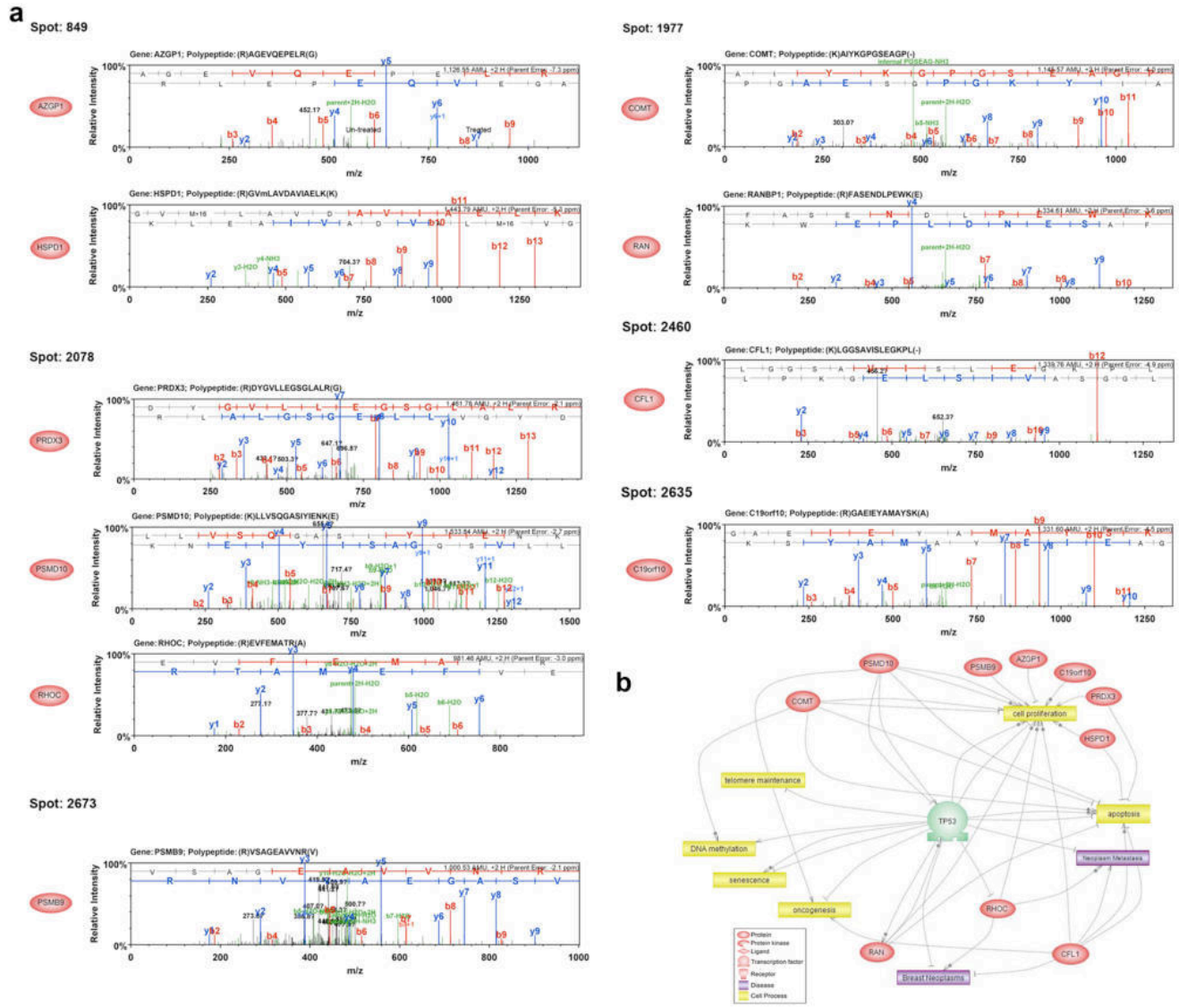
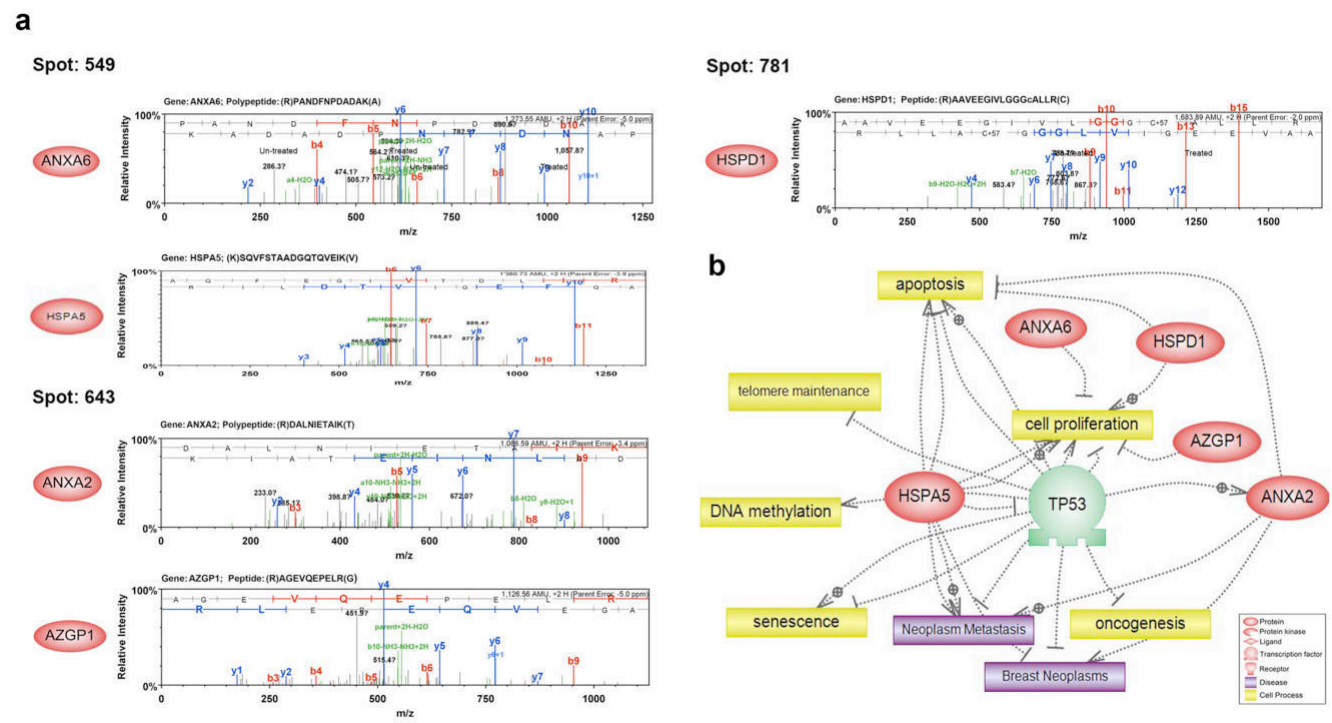

a
Spot: 849
Gene: AZGP1; Polypeptide: (R)AGEVQEPELR(G)
AZGP1
Gene: HSPD1; Polypeptide: (R)GVmLAVDAVIAELK(K)
HSPD1
Spot: 2078
Gene: PRDX3; Polypeptide: (R)DYGVLLEGSGLALR(G)
PRDX3
Gene: PSMD10; Polypeptide: (K)LLVSQGASIYIENK(E)
PSMD10
Gene: RHOC; Polypeptide: (R)EVFEMATR(A)
RHOC
Spot: 2673
Gene: PSMB9; Polypeptide: (R)VSAGEAVVNR(V)
PSMB9
Spot: 1977
Gene: COMT; Polypeptide: (K)AIYKGPGSEAGP(-)
COMT
Gene: RANBP1; Polypeptide: (R)FASENDLPEWK(E)
RAN
Spot: 2460
Gene: CFL1; Polypeptide: (K)LGGSAVISLEGKPL(-)
CFL1
Spot: 2635
Gene: C19orf10; Polypeptide: (R)GAEIEYAMAYSK(A)
C19orf10
Relative Intensity
m/z
b
PSMD10
PSMB9
AZGP1
C19orf10
COMT
PRDX3
cell proliferation
HSPD1
telomere maintenance
TP53
apoptosis
DNA methylation
Neoplasm Metastasis
senescence
oncogenesis
RHOC
RAN
CFL1
Breast Neoplasms
Protein
Protein kinase
Ligand
Transcription factor
Receptor
Disease
Cell Process

Figure 6.


a
Spot: 549
ANXA6
Gene: ANXA6; Polypeptide: (R)PANDFNPDADAK(A)
HSPA5
Gene: HSPA5; (K)SQVFSTAADGQTQVEIK(V)
Spot: 643
ANXA2
Gene: ANXA2; Polypeptide: (R)DALNIETAIK(T)
AZGP1
Gene: AZGP1; Peptide: (R)AGEVQEPELR(G)
Spot: 781
HSPD1
Gene: HSPD1; Peptide: (R)AAVEEGIVLGGGcALLR(C)
Relative Intensity
m/z
Un-treated
Treated
b
apoptosis
ANXA6
HSPD1
telomere maintenance
cell proliferation
AZGP1
DNA methylation
HSPA5
TP53
ANXA2
senescence
Neoplasm Metastasis
oncogenesis
Breast Neoplasms
Protein
Protein kinase
Ligand
Transcription factor
Receptor
Disease
Cell Process

Dataset S1

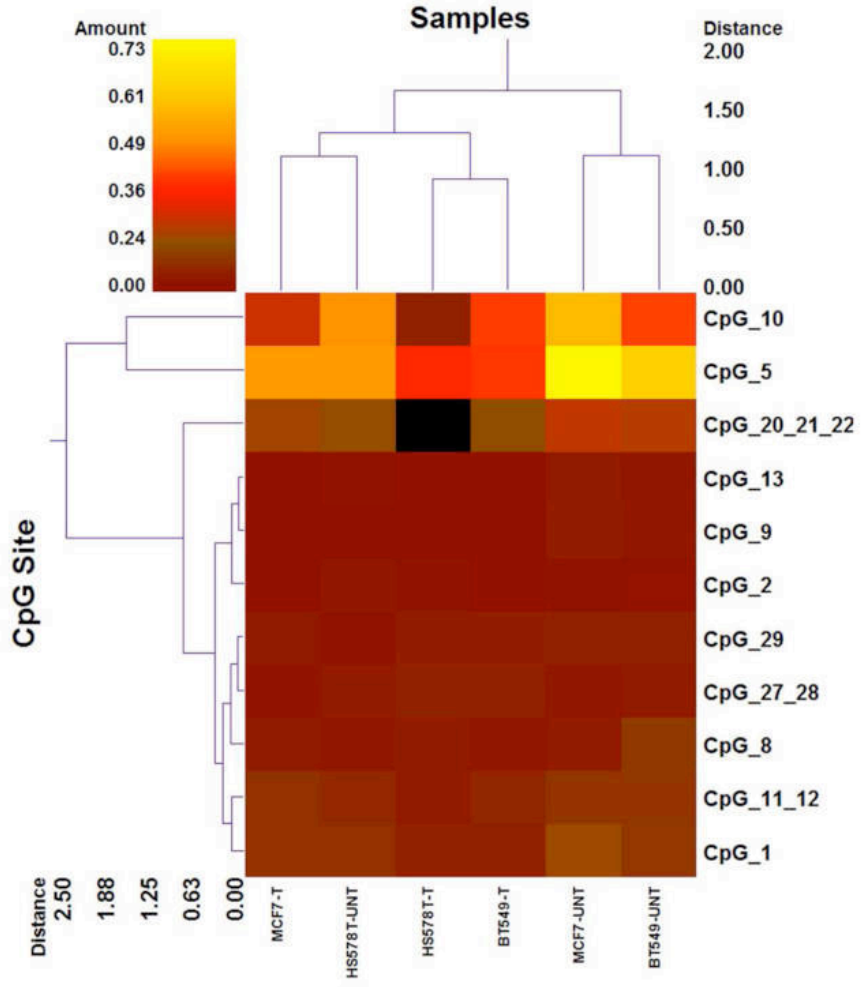
Table 1. The sequence of PCR tagged primers for in vitro transcription.

Gene	Primer	Sequence (5'→3')	Length	Ta	Prodoct Size (bp)
CDKN1A	tag-EN1-FW	AGGAAGAGAGGGTAAATTTTTGTTTGTTAGAGTGG	25+10	60	419
	T7-EN1-RV	CAGTAATACGACTCACTATAGGGAGAAGGCTTAACTTCRACAACTACTCACACCT	24+31		

Table 2. The sequence of PCR primers for TP53 gene sequencing.

Gene		Primer	Sequence (5'→3')	Length	T_a	Prodoct Size (bp)
TP53	Exon-5	Forward	GTTTCTTTGCTGCCGTCTTC	20	58.00	365
		Reverse	AGCCCTGTCGTCTCTCCAG	19		
	Exon-6	Forward	GAGACGACAGGGCTGGTTG	19	59.00	213
		Reverse	TAACCCCTCCTCCCAGAGAC	20		
	Exon-7	Forward	CCTGCTTGCCACAGGTCTC	19	58.00	271
		Reverse	GTATGGAAGAAATCGGTAAG	20		
	Exon-8	Forward	GCCTCTTGCTTCTCTTTTCC	20	58.00	329
		Reverse	TAACTGCACCCTTGGTCTCC	20		
mtDNA	D-Loop	Forward	CCCCATGCTTACAAGCAAGT	20	58.00	982
		Reverse	GCTTTGAGGAGGTAAGCTAC	20		

For the PCR on bisulfite-treated genomic DNA (gDNA) and each exon of TP53 , the following PCR conditions were used: 1x: 95°C for 10 min; 48x: 95°C for 30s, Ta for 40s, 72°C for 1 min; 1x 72°C for 5 min. The PCR cocktail was: 2μL DNA (2.00μL of at least 10 ng/μL DNA for a final concentration of 2ng/μL per reaction) in a 10μL total volume using 10 pmol of each primer, 200μM dNTP, 0.2 unit Hot Start Taq DNA polymerase, 1.5mM MgCl2 and the buffer supplied with the enzyme.



S1. Double dendrogram presents the methylation profiles of P21 for the studied cell lines before and after DAC treatment (Red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0-100).

3.6 Under-review manuscript

Dysregulation of P53 Pathway-Associated microRNAs in Breast Cancer

Author: Zeinab Barekati, Ramin Radpour, Martin M. Schumacher, Nicole Hartmann, Fabio levi, Paolo Toniolo, Per Lenner, Frank Staedtler, Xiao Yan Zhong

Summary: Present study investigated differentially expressed miRNAs that led to dysregulation of the p53 signaling pathway in breast cancer. The results reported in this study increase the understanding of impaired p53 pathway by miRNAs in human breast cancer.

Author contributions: Zeinab Barekati was involved in experimental design, performing the study, data analysis, interpreting data and writing the manuscript.

Running title: P53 and miRNA dysregulation

Dysregulation of P53 Pathway-Associated microRNAs in Breast Cancer

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Prof. Dr. Xiao Yan Zhong

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Abstract

Loss of p53 function and the impaired p53 signalling pathway have been reported in many types of cancer. Present study investigated differentially expressed miRNAs, which associated with dysregulation of the p53 signalling pathway on the basis ofP53 status and in-*silico* pathway analysis. We identified aberrant expression of a set of miRNAs impairing the p53 signalling pathway in breast cancer cell lines (miR-125b, miR-15a, miR155, miR-16, miR-183 and miR-21; ≥2 Fold change). Additionally, we assessed the expression pattern of these miRNA candidates in clinical specimens. The results showed significant dysregulation of miR-125b and miR-21 in tumor tissues compared to the normal matched tissues in the wt-p53 breast cancer patients ($P<0.05$). Indeed, the conditional expression of miR-21 toP53 status has been indicated ($P<0.05$).

In conclusion, the results reported in this study increase the understanding of impairedp53 pathway by miRNAs in human breast cancer and suggest that aberrant over-expression of miR-21 as an important marker for the pathogenesis of wt-p53 breast cancer.

Key Words: Breast cancer, P53, miRNA, mutation.

Introduction

Breast cancer is the most common diagnosed cancer with high mortality rate among females worldwide (1). Breast cancer is associated with sequential accumulation of genetic, epigenetic and molecular abnormalities leading to deregulation of tumor suppressor genes and activation of oncogenes. P53, a tumor suppressor gene, is an important key in prevention of neoplastic development by regulating cell cycle progression and apoptosis as well as maintaining genetic stability in response to cellular stresses (2, 3). Thep53 pathway consists of many genes, involving themselves in different biological outcomes, interacting with other signalling pathways and acting as autoregulatory feedback loops in response to p53 activation (4-6). Loss of p53 function and impaired p53 signalling pathway have been reported in many types of cancer(7).

Inactivation of p53 function can be recognized by mutation and other molecular mechanisms. P53 is mutated in over half of the human cancers and tend to be observed in DNA binding domain at exons 5-8 (8, 9). However, the prevalence of *P53* mutations in breast cancer is considerably lower (~ 20%) than the other type of cancers (9). Thep53 pathway dysregulation, independent of mutation, provided an insight into the other responsible mechanisms such as microRNAs (miRNAs) network that might play a role in impairing the p53 signalling pathway in breast cancer.

miRNAs are a class of small non-coding regulatory RNA, which is synthesized by RNA polymerase II. Mature miRNAs, 19-25 nucleotide in length associated with RNA-induced silencing complex (RISC) and bind to the complementary protein coding messenger RNA (mRNA) target that eventually mediate gene expression modulation (10). Several reports have demonstrated important roles for miRNA in different biological processes. Recently, aberrant expression of miRNAs has been identified to the pathogenesis of different

human cancers (11-13). It has been shown that deregulated miRNAs contribute to the initiation and progression of cancer by altering the expression of crucial pathways involved in cell proliferation and survival (14, 15).

Numerous studies have been directed toward understanding the cross talk between *P53* transcription regulation and miRNAs expressions. The impact of miRNAs in the dysregulation of p53 and its signalling pathway in solid tumors has been studied, however, is not well characterized (16, 17). Here, we report a set of miRNAs that impairs thep53 pathway depending onP53 status in breast cancer by performing miRNA expression profiling linking to aberrant mRNA expression of thep53 pathway.

Material and methods

Cell lines and clinical samples

Human breast cancer cell lines MCF7 (wt-p53), HS578T and BT549 (mutant p53) (18) has been subjected for this study. The characteristics and media conditions for each cell line are summarized in the table 1.

The clinical samples obtained from UMASS Cancer Center Tissue Bank with the approval of the institutional Review Board (IRB) of UMASS Medical School. The studied cohort consisted of 60 samples including tumor tissues and matched normal tissues from 30 patients with invasive ductal carcinoma. The clinical characteristics such as staging, age, histological grading and hormone receptor expression from the breast cancer patients are listed in Table 2.

DNA, RNA, miRNA isolation

All the studied samples were subjected to simultaneous isolation of DNA, RNA and miRNA using AllPrep® DNA/RNA/Protein Mini Kit (QIAGEN AG, Basel, Switzerland).

For the cell lines 5×10^6 cells per sample and for the clinical samples 2mg frozen tissues were used to extract molecular species according to the published protocol (19). The RNeasy MinElute Cleanup Kit (QIAGEN AG, Basel, Switzerland) was used for purification of enriched miRNAs. The quantity of extracted molecular species was assessed using a NanoDrop ND-1000 spectrophotometer (Biolab, Mulgrave, VIC, Australia). Extracted RNA samples were analyzed for the size fractionation using RNA 6000 Nano LabChip® and for the enriched miRNAs (Agilent Technologies, GmbH, Waldbronn, Germany) using RNA 6000 Pico LabChip®.

Mutation analysis of *TP53*

The studied cohort was screened for the integrity of *P53* gene using direct sequencing of exons 5-8 as described in the previous work (20). Direct DNA sequencing was performed by using a Big Dye terminator v3.1 cycle sequencing kit and automated sequencer was performed (ABI 3130, Applied Biosystem). The DNA sequencing results were compared with the reference sequences from the GeneBank (www.ncbi.nlm.mih.gov/nucleotide) using the DNASTAR sequence alignment software (DNASTAR Lasergene 8, Inc., Madison, USA). All sequencing reactions were performed in both directions and confirmed for concordance.

MicroRNA expression analysis

Microarray analyses of the three human breast cancer cell lines enriched miRNA were carried out using Affymetrix GeneChipR miRNA array (v1.0) according to manufacturer's recommended protocol (21). Using Partek Genomics Suite software v6.5 (Partek Incorporated, Missouri, USA), differentially expressed miRNAs were then defined after log transformation and Robust Multi-array Analysis (RMA) normalization with two additional stringent filtering criteria (mean intensity greater than six and fold change greater than two) (21).

Unsupervised hierarchical clustering of significant up/down-regulated miRNAs was applied using Partek software with standard Pearson's correlation as a similarity measurement, and Ward'd method for clustering the data. All miRNA microarray data compiled for this study have been made publicly available on GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE28969.

For clinical samples miRNA expression was measured using quantitative Real-Time PCR (qRT-PCR). The complementary DNAs were synthesized using miScript Reverse Transcript kit (QIAGEN AG, Basel, Switzerland). The miScript SYBR Green PCR kit and commercially available primers for mature miRNAs (QIAGEN AG, Basel, Switzerland) were used to perform the qRT-PCR (Supplementary data 1, TableS1). The miRNA expression was normalized using the geometric mean of two reference genes recommended by Qiagen (considering the lowest variation in all the analyzed samples).

To validate the microarray findings, the qRT-PCR was performed for the six candidate miRNAs linked to thep53 pathway (supplementary data1).

The p53 signalling pathway PCR Arrays

The synthesis of cDNA was carried out with a starting amount of approximately 100 ng total RNA using RT^2 first strand kit (SABioscience, Fredrick, Md USA). The quantitative RT-PCR array was performed on 84 genes involved in the human p53 signalling pathway according to the manufacturer's protocol (SABioscience, Fredrick, Md USA) using ABI Prism 7000 Sequence Detection System (Applied Biosystems). The complete list of the genes contained in human p53 signalling pathway PCR array is presented in the following link: http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-027A.html.

The accurate expression levels were measured using the comparative Ct method (22). The target genes were normalization by the geometric mean of two reference genes (*ACTB* and *GAPDH*) with the stable expression among the samples (23).

The expression differences obtained by PCR arrays were verified by qRT-PCR for *CDKN2A*, *MDM2*, *PTEN* and *TP53* selected genes in duplicate. The fold changes were measured relative to the wt-p53 cell line (MCF-7) (supplementary data1).

In silico prediction of miRNA targets and pathway analysis

Conserved and non-conserved targets of detected miRNAs were identified using TargetScan 5.1 (http://www.targetscan.org/) and RNA22 program (http://cbcsrv.watson.ibm.com/rna22.html). For comparing different kinds of cellular interactions, including protein-protein interactions as well as genes-miRNAs interactions, the Pathway Studio® software version 7 and ResNet® 7 (Mammal) database (Ariadne Genomics, Inc., Rockville, USA) was applied. The data set containing gene/miRNA identifiers and corresponding fold changes were uploaded into Pathway Studio and each gene identifier was mapped to its corresponding gene object.

Statistical analysis

Data analysis was performed using the SPSS software (Statistical Software Package for Windows, version 17). Distribution of data was analyzed by Kolmogorov-Smirnov. The Mann-Whitney U test was used to determine the significance level of miRNA expression in clinical samples and Spearman's rho test were performed to find significant correlations. A probability of $P<0.05$ was considered to be statistically significant.

Results

MicroRNA and p53 signalling pathway expression profiling

Gene expression results obtained from Microarray chip were analyzed according to theP53 status between breast cancer cell lines. The comparison of gene expression pattern of wt-p53 (MCF-7) and the mutant p53 (BT549 and HS578T) breast cancer cell lines revealed 59 differentially expressed miRNAs (≥2 fold change). The annotated list of the differentially expressed miRNAs is provided in supplementary data 2. The hierarchical cluster expression profile of 59 differentially expressed miRNAs between all three cell lines is illustrated in figure 1.

The comparison of p53 signalling pathway expression analysis, in BT549, HS578T and MCF-7 cell lines, showed different expression pattern in 64 genes with more than (≥2 Fold change). From the 64 genes, twenty-seven genes (intersection genes) were differentially expressed in both BT549 and HS578T breast cancer cell lines compared to MCF-7 cell line. The union list of genes consisted of 31 for BT549 and 6 genes for HS578T, indicating higher difference between BT549 and MCF-7 (Fig.1, Supplementary data 2). The complete expression pattern of the p53 signalling pathway by hierarchical cluster analysis within the three studied cell lines is presented in figure1.

The differentially expressed miRNAs in the three studied cell lines were analyzed with pathway studio to find their relation to the differentially expressed intersection genes of p53 signalling pathway. This analysis revealed six miRNAs (miR-125b, miR-15a, miR155, miR-16, miR-183 and miR-21) that are linked to deregulation of thep53 pathway. From the six identified miRNAs; miR-125b, miR-183 and miR-21 showed over-expression in the MCF-7 cell line whereas miR-15a, miR155 and miR-16 had higher expression level in the BT549 and HS578T(≥2 Fold change). The expression of the six identified miRNAs

significantly correlated to the aberrant expression of mRNAs (*BCL2*, *CDC25A*, *MLH1*, *MSH2*, *PRKCA1*, *TP53*, *SESN1*; ≥2 Fold change, $P<0.05$) contributed in p53 signalling pathway in aP53 status-dependent manner (Fig1.). The results showed over-expression for BCL2, *CDC25A*, *MLH1*, *MSH2*, *TP53*, *SESN1* and lower-expression for *PRKCA1* in the MCF-7 cell line compared to the BT549 and HS578T cell lines (≥2 Fold change).

The expression profile of the six identified miRNAs and the p53 signalling pathway PCR arrays are technically confirmed using qRT-PCR (Supplementary data 1.)

***TP53* status and identification of the microRNA candidates in clinical samples**

The *TP53* mutation analysis in paired tumor and matched normal tissues has been compared with the *TP53* GenBank sequence (NC_000017 [gi:224589808]). The sequence analysis of exons 5-8 revealed mutations in the 6 patients (20%) of the studied cohort. From six mutant cases, four of them identified with somatic mutations and two other cases with germline mutations. The identified mutations were localized in exons 6 and 7. The details about the *TP53* mutation analysis results are presented in Table 3. The sequencing results of the detected mutations are provided in supplementary data 3.

The six miRNA candidates linking to the p53 signalling pathway were analyzed in breast cancer patients. The relative miRNA expression levels of tumor tissues were compared to the matched normal tissues. The results showed significant down-regulation for miR-125b whereas miR-21 was up-regulated in cancer tissues ($P<0.05$, Fig.2). The miRNAs expression for miR-15a, miR155, miR-16 and miR-183 revealed slight fluctuation in the tumor tissues when compared to the matched normal tissues, however, these alterations did not reach to the statistical significance level.

Further analysis of the significant dysregulated miRNAs, miR-125b and miR-21, based onP53 status in the studied cohort showed significant over-expression for miR-21 in wt-p53 breast cancer patients ($P<0.05$, Fig.3) , however, miR-125-b did not significantly differ between wt-p53 and mutant p53 breast cancer patients.

A negative correlation between up-regulation of miR-21 and down-regulation of miR125-b was found ($P<0.001$, Fig.4). The relationship between clinicopahological parameters and dysregulation of miR-125b and miR-21 were studied. The analysis indicated an inverse correlation between over-expression of miR-21 and lymph node involvement as well as bigger tumor size in the present cohort ($P<0.05$, Spearman's rho test).

Discussion

A wide range of cancers has been linked to the dysfunction of p53 and deregulation of the p53 signalling pathway that could be rationalized by a complex of genetic, epigenetic and molecular abnormalities. The frequency of *P53* mutations is low in breast cancer (7, 9), which suggests other molecular abnormalities in particular aberrantly expressed microRNAs may change the properties of p53 tumor suppressor gene and its signalling pathway independent of mutation.

The comparison of miRNA expression profiling of the wt-p53 breast cancer cell line (MCF-7) and mutant p53 breast cancer cell lines (BT549, HS578T) along with their interactions to the p53 signalling pathway revealed differential expression of miR-125b, miR-15a, miR155, miR-16, miR-183 and miR-21 ($\geq$2 Fold change). The dysregulation of the identified miRNAs in cancer has been verified in the previous reports, having roles in cancer progression (miR-125b, miR-15a and miR-16), invasion and metastasis (miR-155, miR-183 and miR-21) and they have suggested with prognostic values (24-28). The *in-silico* pathway analysis demonstrated potential links between dysregulation of the miRNA candidates and

aberrant expression signature of their target, *BCL2*, *CDC25A*, *MLH1*, *MSH2*, *PRKCA1*, *TP53* and *SESN1,* genes. These genes play role in variety of biological responses including apoptosis (*BCL2*), cell cycle regulation and proliferation (*CDC25A, PRKCA1, SESN1*)(29-32) and DNA repair (*MLH1*and *MSH2*) (33). The association of malfunction of those genes and defective cell responses are well describe in cancers (32, 34-36). Our finding suggested miR-125b, miR-15a, miR155, miR-16, miR-183 and miR-21 as disruptors ofp53 pathway in breast cancer.

Studying the miRNA disruptors in clinical specimens revealed significant dyregulation of miR-125b and miR-21 in the tumor tissues compared to the normal matched tissues ($P<0.05$), which wasP53 status-dependent manner for miR-21 ($P<0.05$). The pathway analysis and previous reports presented regulatory role for over-expressed miR-125b on p53 post-transcriptional modification and on the p53 signalling pathway (37, 38). However, current results showed down-regulation of miR-125b in patients with invasive ductal carcinoma, which are in line with previous studies in breast cancer (38-40).

The high expression of miR-21 has been indicated in various cancers and it is suggested as an oncomiR (14, 41, 42). Several studies demonstrated that induction of miR-21 expression could elevate tumor cell proliferation, migration and invasion in cancers (43, 44). Few putative targets of miR-21 have been experimentally validated though computational analysis predicted hundreds mRNAs with complementary or partial complementary sites at 3'UTR. In our study, pathway analysis presented regulatory connection between miR-21 and *TP53*. Indeed, the significant over-expression of miR-21 in both the wt-p53 patients with invasive ductal carcinoma and the wt-p53 human breast cancer cell line suggested conditional expression of miR-21 toP53 status. However, the binding site in the p53 mRNA for miR-21 has not yet been characterized and regulation of p53 by miR-21 or vice versa is a controversial issue (45, 46) and needed to be investigated in more depth.

In conclusion, the results reported in this study increase the understanding of the impairedp53 pathway via a subset of miRNAs in human breast cancer and suggest that aberrant over-expression of miR-21 may be important for the pathogenesis of wt-p53 breast cancer .

Acknowledgements

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Conflicts of Interest

There is no Conflict of Interest in the present study.

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Table 1. Human breast cancer cell lines characteristics.

Cell line	TP53	Source	Tumor Type	Culture media
MCF7	+/+WT	PE	IDC	DMEM
BT549	++M	P.Br	IDC, pap	RPMI
HS578T	++M	P.Br	IDC	DMEM

P.Br, primary breast; PE, pleural effusion. TP53 status: TP53 mutational status obtained from exon 5-8 analysis (M, mutant; WT, wild-type protein). Media conditions: FBS, fetal bovine serum (10%); cultured at 37°C and 5% CO_2; DMEM, Dulbecco's modified Eagle's medium, GIBCO #11965-092; RPMI, RPMI medium 1640, GIBCO #27016-021.

Table 2. Clinical characteristics of the studied patients with Invasive Ductal Carcinoma.

Total no. of patients	Pathologic stage			Age mean ± S.D. (range)	Histological grade			Tumor size				Nodes involvement	ER positive patients	PR positive patients	C-ERB2 positive patients
	I	II	III		1	2	3	T1	T2	T3	T4				
30	10	10	10	60.7±13.83 (36-94)	3	16	11	13	13	2	2	18	26	20	1

ER, Estrogen Receptor; PR, Progestron Receptor; C-ERB2, Herceptin Receptor.

Table 3. *TP53* mutation analysis results of Breast Cancer patients.

Number	Location	Nucleotide change	Mutation Status	Mutation Type	Observed in patient (No.)
1	Exon 6	c.12601,C>T	Heterozygous	Somatic	1
2	Exon 6	c.12654,A>G	Heterozygous	Germeline	1
3	Exon 6	c.12674, A>G	Heterozygous	Somatic	1
4	Exon7	c.13382, C>T	Heterozygous	Germeline	1
5	Exon7	c.13437, C>T	Homozygous Heterozygous	Somatic Somatic	2

Figure legend

Figure 1. A and B) Gene and miRNA expression profiles of the three studied genes. a1) the total number of differentially expressed genes in BT549 and HS578T cell lines compared to MCF-7 cell line, a2) Hierarchical cluster analysis of the p53 signalling pathway gene expression. b1) the total number of differentially expressed microRNA in BT549 and HS578T cell lines compared to MCF-7 cell line, b2) Hierarchical cluster analysis of the differentially expressed miRNAs in BT549 and HS578T cell lines compared to MCF-7 cell line. C) Pathway analysis of the differentially expressed miRNAs linked to the differentially expressed p53 signalling pathway genes.

Figure 2. The fold change differences of the six identified miRNAs linked the P53 signalling pathway of the tumor tissues compared to the matched normal tissues, *; $P<0.05$.

Figure 3. The miRNAs expression according to theP53 status in patients with invasive ductal carcinoma. a) The fold change difference of miR-21in P53 wild type and mutant breast cancer patients ($P<0.05$). b) The fold change difference of miR-125b in P53 wild type and mutant breast cancer patients.

Figure 4. The correlation study between miR-21 and miR125-b expression ($P<0.001$).

Figure 1.

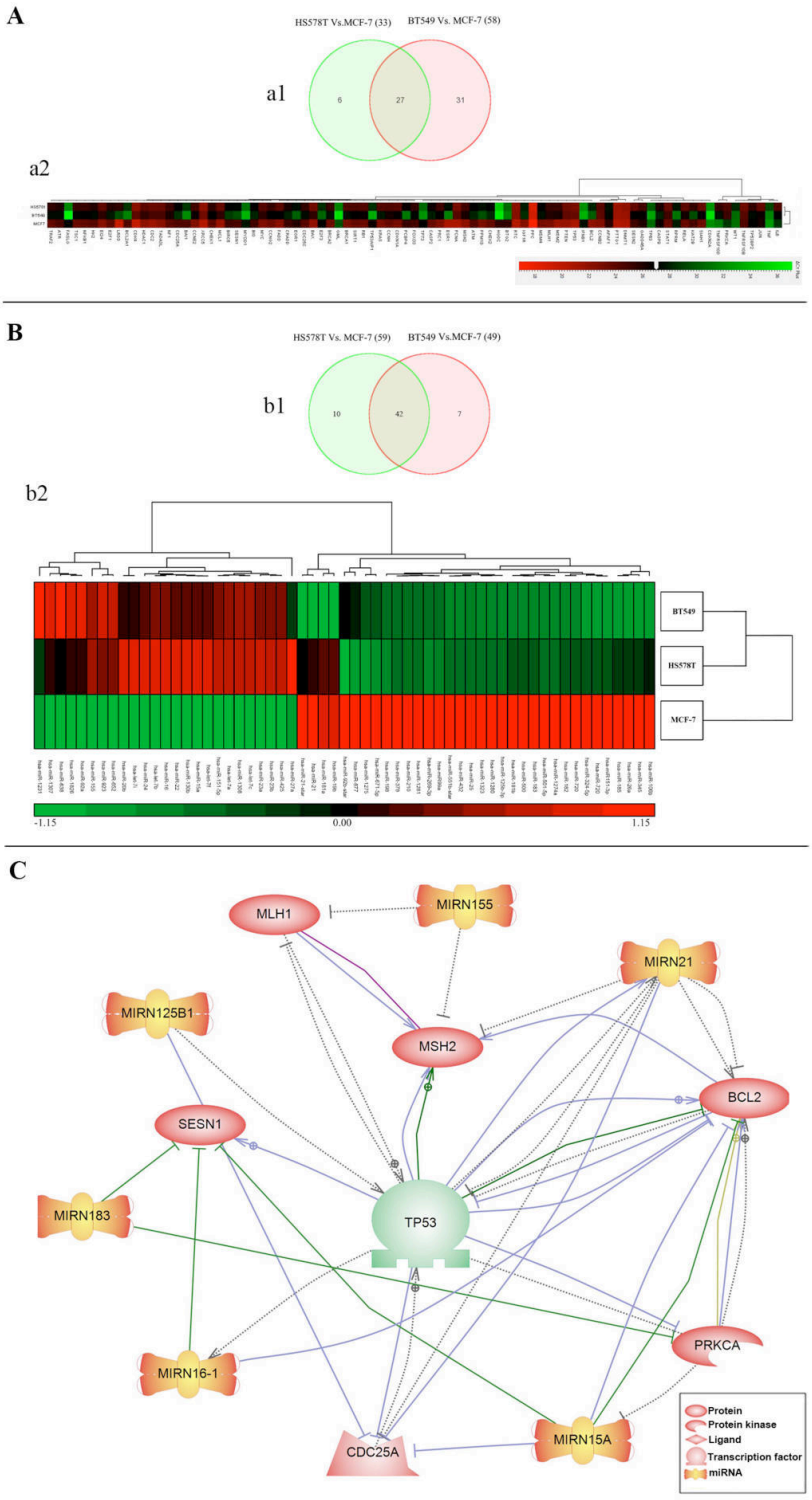

A
HS578T Vs.MCF-7 (33)
BT549 Vs. MCF-7 (58)
a1
6
27
31
a2
B
HS578T Vs. MCF-7 (59)
BT549 Vs.MCF-7 (49)
b1
10
42
7
b2
BT549
HS578T
MCF-7
-1.15
0.00
1.15
C
MLH1
MIRN155
MIRN21
MIRN125B1
MSH2
BCL2
SESN1
MIRN183
TP53
PRKCA
MIRN16-1
CDC25A
MIRN15A
Protein
Protein kinase
Ligand
Transcription factor
miRNA

Figure 2.

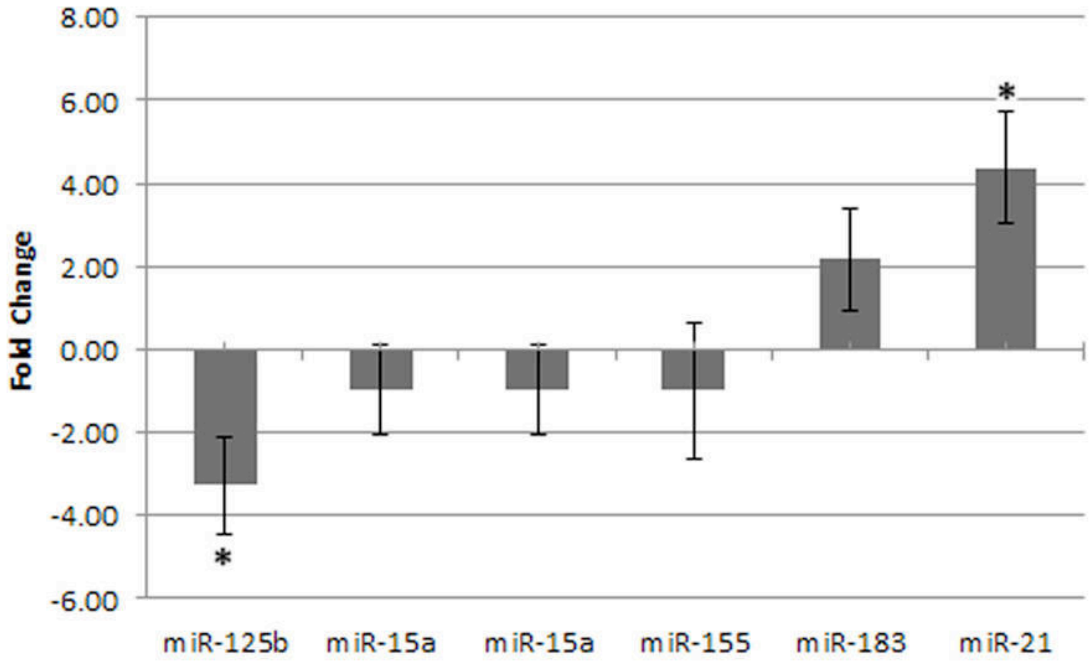

8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
-6.00
Fold Change
*
*
miR-125b
miR-15a
miR-15a
miR-155
miR-183
miR-21

Figure 3.

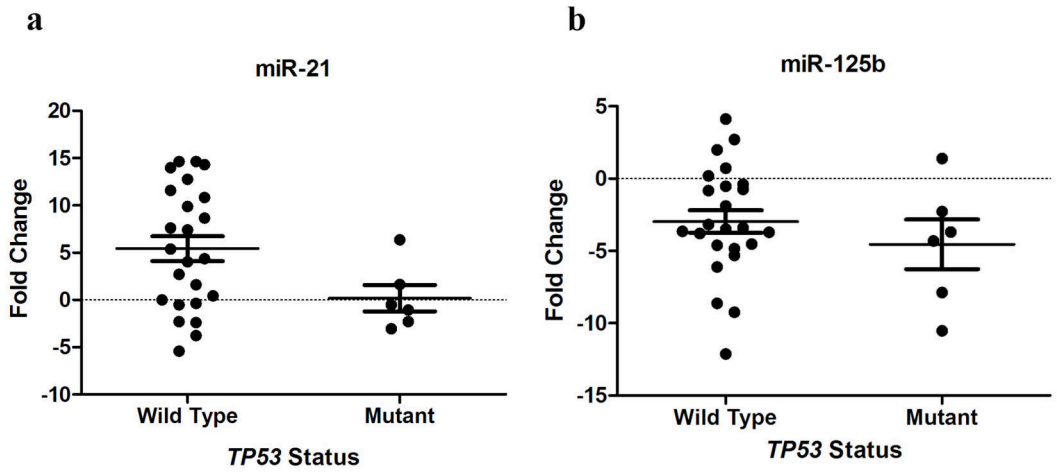
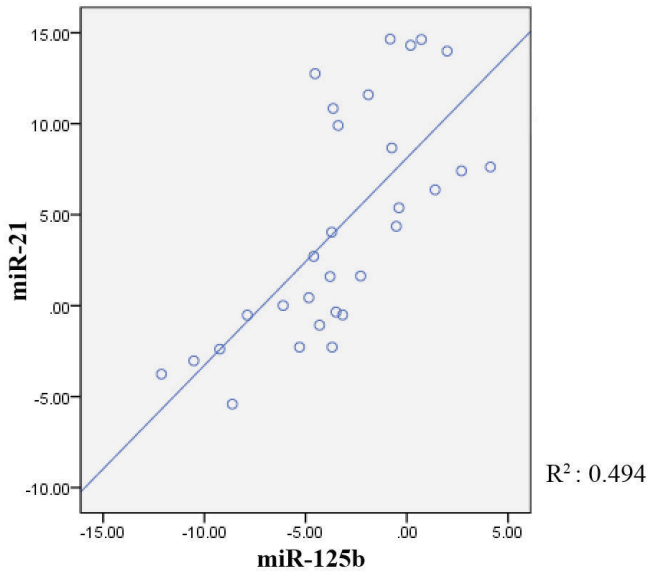


Figure 4.



R^2 : 0.494

Supplementary Data 1.

Quantitative Real Time PCR (qRT-PCR) validation.

To confirm the microarray findings, qRT-PCR was carried out for six candidate miRNAs (miR-125b, miR-15a, miR155, miR-16, miR-183 and miR-21). For each cell line, the same extracted miRNA for microarray analysis was applied to synthesize the cDNA using miScript Reverse Transcript kit (QIAGEN AG, Basel, Switzerland). The qRT-PCR for detection of miRNAs was performed using miScript SYBR Green PCR kit with the commercially available primers for mature miRNAs (QIAGEN AG, Basel, Switzerland) (TableS1). Raw values were normalized using geometric mean of two recommended reference genes by the company (Hs-RNU5A and Hs-RNU6B) with the lowest variation in all the analyzed samples.

Validation of PCR arrays were done by performing qRT- PCR of four candidate mRNAs (*CDKN2A*, *MDM2*, *PTEN* and *TP53*). The new cDNAs were synthesized from the same extracted mRNA samples using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Real-time primers were then designed for each gene using Primerquest Software (Integrated DNA Technologies). For qRT-PCR analysis, synthesized cDNAs amplified with specific gene primers using SYBR® Green 2X PCR Master Mix (Applied Biosystems, UK). Delta Cts were calculated using geometric mean of two reference genes (*GAPDH*, *B2M*).

Real-time PCR reactions for the both miRNA and mRNA expression analysis were performed in two replicates and including no-template controls using ABI Prism 7000 Sequence Detection System (Applied Biosystems). The fold difference for each sample was calculated using Livak method and relative to MCF-7 cell line with wild type p53 status (Fig S1, and b) [1].

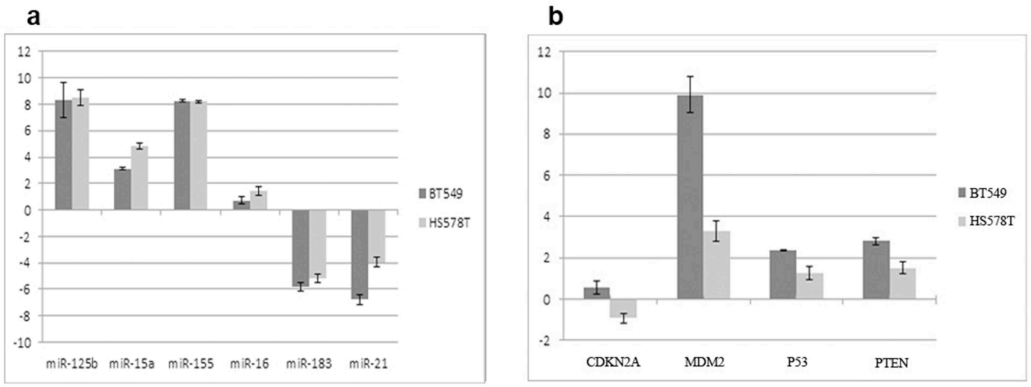


Figure S1. Validation of the relative expression of miRNAs and mRNAs in BT549 and HS578T compared to MCF-7. a) The relative expression pattern of miRNAs linked to deregulation of P53 signaling pathway. b) The relative expression pattern of selected mRNAs from P53 signaling pathway.

Table S1. The primer sequence and ID of selected miRNAs

Mir ID	Sanger ID	Sanger accession	Sequence 5‘ → 3‘
Mir 155	hsa-miR-155	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU
Mir 125b	hsa-miR-125b	MIMAT0000423	UCCCUGAGACCCUAACUUGUGA
Mir 15a	hsa-miR-15a	MIMAT0000068	UAGCAGCACAUAAUGGUUUGUG
Mir 16-1	hsa-miR-16	MIMAT0000069	UAGCAGCACGUAAAUAUUGGCG
Mir 21	hsa-miR-21	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA
Mir183	hsa-miR-183	MIMAT0000261	UAUGGCACUGGUAGAAUUCACU
RNU5A-1	hsa-miR-26a	MIMAT0000082	UUCAAGUAAUCCAGGAUAGGCU
Mir 24	hsa-miR-24	MIMAT0000080	UGGCUCAGUUCAGCAGGAACAG

Table S2. The primer sequence of the selected mRNA for qRT-PCR

Gene name	Ensembl Gene ID	Primer	Sequence	Length (bp)	T_a	Product size
ACTB	ENSG00000075624	FW	CGTCTTCCCCTCCATCGT	18	60	181
		RV	GGTGTGGTGCCAGATTTTCT	20		
B2M	ENST00000349264	FW	GTGCTCGCGCTACTCTCTCT	20	60	150
		RV	GTCAACTTCAATGTCGGATGG	21		
CDKN2A	ENST00000530628	FW	CAGGTTCTTGGTGACCCT	18	60	193
		RV	CATCATGACCTGGTCTTCTAGG	22		
MDM2	ENST00000462284	FW	CCTGGTTAGACCAAAGCCA	19	60	244
		RV	CCAAGTTCCTGTAGATCATGG	21		
PTEN	ENTT00000371953	FW	CGGAACTTGCAATCCTCAG	19	60	233
		RV	GTTTCCTCTGGTCCTGGT	18		
P53	ENST00000445888	FW	CTCCTCAGCATCTTATCCGA	20	60	227
		RV	CCAGTAGATTACCACTGGAGTC	22		

Reference

1. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods* 2001, **25**(4):402-408.

Supplementary data3.

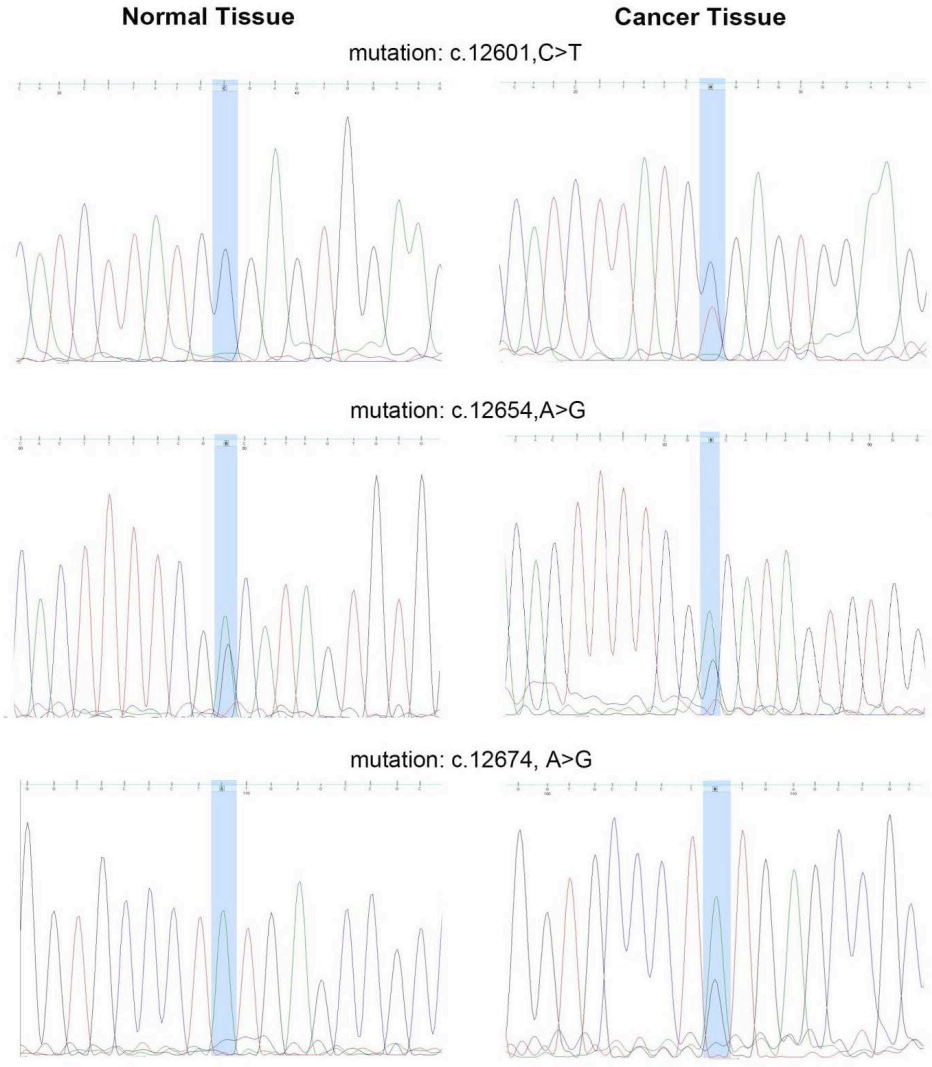


Figure. S1. Sequencing results of the detected *TP53* mutations at Exon 6 in the cancer tissues compared to the normal tissues.

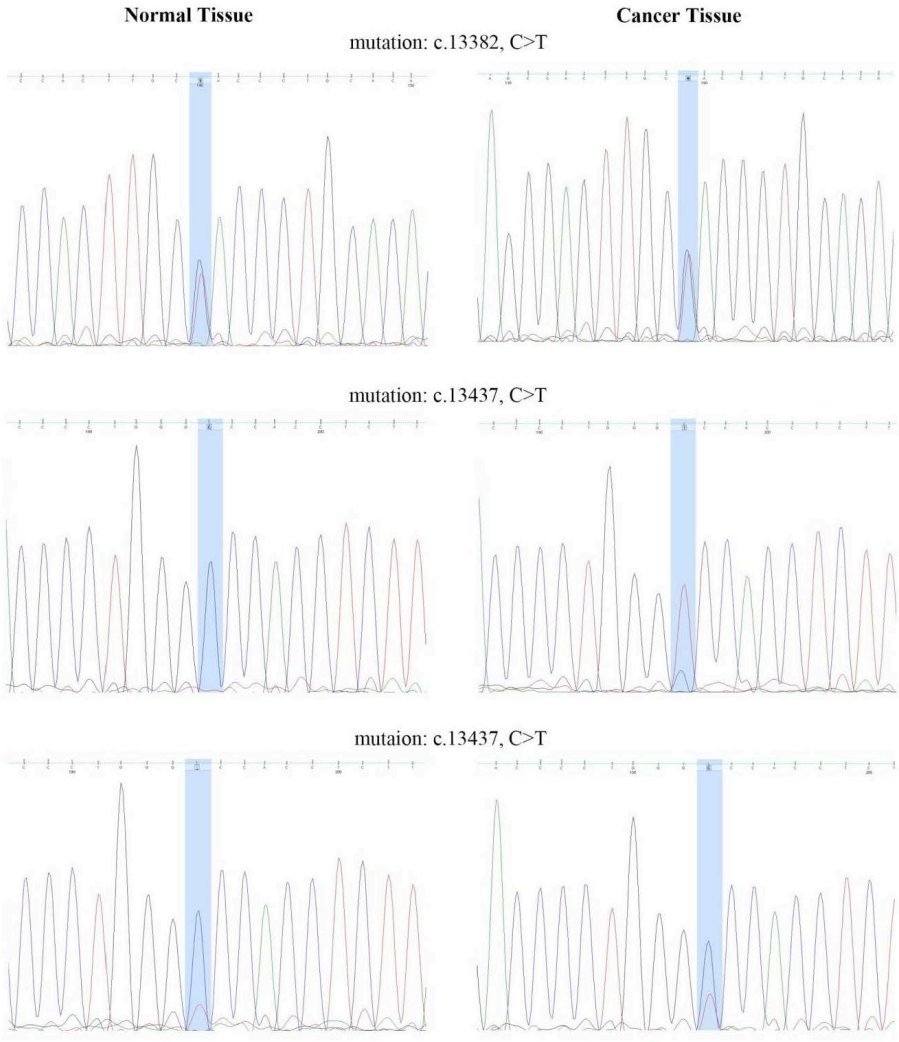


Figure. S2. Sequencing results of the detected *TP53* mutations at Exon 7 in the cancer tissues compared to the normal tissues.

4. SUMMARY OF THE RESULTS

4.1 Mechanism of p53 inactivation independent of *TP53* mutations

4.1.1 Quantitative methylation profiles of the p53 auto-regulatory feedback loops

We analyzed the methylation patterns of *P14^{ARF}/MDM2/TP53* and *PTEN* in 66 triple-matched samples (cancerous tissues, matched normal tissues and serum samples) from 22 wild type p53 (wt- p53) breast cancer patients. For all the genes, one amplicon per gene and in total 117 CpG sites per sample (total of 7020 sites in 66 analyzed samples) were analyzed (Table 1 and Supplementary Material 3; section 3.3). Hierarchical cluster analysis profiling of the promoter alterations of the four studied genes is shown in Figure 2A (Section 3.3).

The quantitative methylation profiling of *MDM2* oncogene and *TP53* for both the tumor and the normal tissues showed a relatively comparable methylation pattern whereas *P14^{ARF}* and *PTEN* presented significantly higher methylation levels in tumor tissue versus normal tissue ($P < 0.05$ and $P < 0.01$, respectively). Indeed, the *PTEN* methylation pattern was significantly higher methylated in the matched serum samples than the normal tissues and it showed significant concordance to methylation profile of the tumor tissues (Spearman's rho test; $P < 0.05$).

The comparison of methylation ratio between the aforementioned genes and their upstream regulatory sequence revealed pronounced accumulation of hypermethylated CpG sites around TATA box region in tumor-derived samples. In serum and normal samples, methylated CpG sites were randomly distributed on 5'-UTRs of the studied genes (Fig. 2C; section 3.3).

4.1.2. The p53-associated proteins upon demethylation treatment

A proteome-based approach in three breast cancer cell lines (MCF7, HS578T and BT549) was performed after treatment with an optimal dosage of 5-aza-2′-deoxycytidine (DAC) and *in-silico* pathway analysis to find out methylated genes with potential link to the P53 pathway. Here, we focus on the results obtained from the wt-p53 breast cancer cell line (MCF-7).

For MCF7, a total of 12 differentially expressed protein spots were detected and 22 proteins were eventually identified by LC-MS-MS analysis (Table 2 and 3; Section 3.4). The in-*silico* pathway analysis demonstrated three up-regulated proteins, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (Atic), calreticulin precursor (Calr) and proliferating cell nuclear antigen (Pcna), which has regulatory role on p53 (Fig.1; Section 3.4).

4.1.3 Aberrant expression of miRNAs impairing p53 pathway

The Gene expression results obtained from Microarray chip were analyzed according to the p53 status of the three studied breast cancer cell lines (MCF7, HS578T and BT549). The comparison of miRNA expression profiling of the wt-p53 breast cancer cell line (MCF-7) and mutant p53 breast cancer cell lines (BT549, HS578T) revealed in total 59 differentially expressed miRNAs (≥2 fold change). Indeed, The mRNA expression profiles of the p53 signaling pathway were measured in the three breast cancer cell lines and followed by *in-silico* pathway analysis of differentially expressed miRNAs. The results displayed association between six differentially expressed miRNAs (miR-125b, miR-15a, miR155, miR-16, miR-183 and miR-21) and dysregulation of *BCL2*, *CDC25A*, *MLH1*, *MSH2*, *PRKCA1*, *TP53*, *SESN*.

Furthermore, the six characterized miRNAs were analyzed in 30 pairs clinical specimens (tumor tissues and matched normal tissues) of breast cancer patients. The results showed significant down-regulation of miR-125b and significant up-regulation of miR-21 in the tumor tissues compared to the normal matched tissues in a *TP53*-dependent expression manner for miR-21 ($P<0.05$, Fig.2 and 3; Section 3.5).

4.2 The relationship between aberrant DNA methylation of p53 related pathway and downstream events

4.2.1 Telomere length shortening and promoter methylation profile of p16/Rb andp53/p21 pathways

For this part, 52 paraffin-embedded breast cancer tissues and 52 paired matched normal tissues were subjected to study. The telomere length in cancer tissues was significantly lower than that in normal tissues (Mann–Whitney U-Test; P<0.001). Quantitative methylation analysis has been performed for *TP53*, *P21*and *P16* genes. One amplicon per gene and 83 CpG sites per sample (4316 CpG sites in total) have been analyzed. The results showed significant correlation between the telomere length shortening in cancer tissues and methylation proportion of *TP53*, *P21* and *P16* (r=-0.33, P=0.001; r=-0.70, P<0.0001 and r=-0.71, P<0.0001; respectively) (Figure 4; Section 3.2).

4.2.2 Mitochondrial DNA alterations and promoter methylation profile of *TP53*/*P14^{ARF}*/*MDM2* and *PTEN*

We assessed Mitochondrial DNA (mtDNA) damage including D-loop region integrity and mtDNA content in breast cancer patients lacking *TP53* mutation. In this context, direct sequencing was performed to analyze D-loop region and the results showed somatic mutations in 36.36% (8 of 22) of studied cancerous samples. The prevalence of detected germline mutations in the D-loop region was markedly higher at 90.91% (20 of 22) than somatic mutations and with mostly multiple germline mutations in each case. The details of the prevalence of germline and somatic mutations are provided in Table 2, and the spectrum of the mutation along the length of the D-loop is illustrated in Figure 3 (Section3.3). The mtDNA contents were significantly lower in the cancer tissues than the normal tissues, with 11.88-fold changes (P< 0.01). In this part of the study we found association between higher methylation proportions of *P14^{ARF}* and *PTEN* in the tumor tissues and mtDNA vulnerability (Section3.3).

4.2.3 The potential link between the p53-associated proteins upon demethylation treatment and mitochondria or telomere maintenance

The proteomics study of the three breast cancer cell lines (MCF7, HS578T and BT549) after treatment with an optimal dosage of DAC and *in-silico* pathway analysis revealed aberrant expression of thioredoxin-dependent peroxide reductase (Prdx3) and proliferating cell nuclear antigen (Pcna) that are linked to mitochondria and telomere maintenance, respectively. The dysregulation of Prdx3 has been found in a p53-dependent status since it was up-regulated in BT549 (mutant p53) cell line and down-regulated in MCF7 (wt-p53) cell line. The overexpression of Pcna protein, involves in telomere maintenance, was detected in MCF7 cell line.

4.3 Contributions of aberrant DNA methylation signature of primary tumor to metastatic lesion

In the current part of the study, methylation ratio on promoter region of *APC, BIN1, BRCA1, BMP6, CST6, ESR-b, GSTP1, P14, P16, P21, PTEN* and *TIMP3* were quantified in matched primary tumors tissues, matched normal tissues and lymph node metastasis of 24 breast cancer patients. For all the genes, one amplicon per gene and 250 CpG sites in total per sample (total of 16,250 sites in 65 analyzed samples) were analyzed (Table 2; Supplementary data 2; Section 3.6). The methylation proportions of *APC, BIN1, BMP6, BRCA1, CST6, ESR-b, P16, PTEN* and *TIMP3* were significantly higher in primary tumor tissue versus matched normal tissue ($P<0.05$). Comparison of methylation statuses of the 12 breast cancer candidate genes in the paired lymph node metastasis to the matched normal tissue showed significantly higher methylation levels for *APC, BMP6, BRCA1* and *P16* genes ($P<0.05$).

The molecular function, biological processes and contribution of the studied genes to developing metastasis were analyzed by ResNet® 7 (Mammal). The pathway analysis revealed that *BMP6*, *BRCA1* and *P16* have a role in prevention of neoplasm metastasis (Fig.3; Section 3.6).

5. FINAL DISSCUSSION AND CONCLUSION

The tumor suppressor gene p53 has an important role in prevention of neoplastic development by regulation of cell cycle progression and apoptosis as well as maintaining genetic stability in response to cellular stress[1, 2]. Loss of p53 function and impaired p53 signaling pathway have been reported in many types of cancer[3]. Dysfunction of p53 mostly reported to be attributed by mutation; however, alterations of upstream and/or downstream regulatory factors could be another plausible mechanism for inactivation of p53 and impairing the p53 signaling pathway in breast cancer. To find molecular mechanisms involved in dysregulation of p53 in breast cancer independent of mutation, we assessed DNA methylation on promoter regions of the p53 auto-regulatory feedback loops (*P14^{ARF}/MDM2/TP53* and *PTEN*). Indeed, dysregulated proteins and miRNAs, which could impair p53-signaling pathway in breast cancer, were characterized. The down-stream events in relation to p53 pathway such as mtDNA and telomere length shortening were studied as well. Furthermore, the contribution of aberrant DNA methylation signature of 12 cancer related genes from primary tumor to matched lymph node metastasis were explored in a patient cohort consisting of invasive ductal carcinoma.

Investigation of DNA methylation profiles of the p53 auto-regulatory feedback loops revealed significant hypermethylation on promoter regions for *P14^{ARF}* and *PTEN* tumor suppressor genes ($P<0.05$), in which hypermethylated CpG sites accumulated around TATA box region. A frequent aberrant methylation patterns of *P14^{ARF}* and a down-regulation of this tumor suppressor gene have been reported in previous breast cancer studies [4, 5]. The hypermethylation of *PTEN* has also been found in breast carcinoma, which resulted in expression repression of a subset of breast cancers [6-8]. Indeed, the significant high methylation proportion of *PTEN* in matched serum samples and its concordance to the tumor tissues ($P<0.05$), proposed a possible use of this gene as a marker in development of blood-based tests. Taken together, present data suggest that the dysregulation of p53 possibly occur by suppression of *P14^{ARF}* and *PTEN* expressions, which emphasizes on the importance of an epigenetic alteration-mediated silencing in the p53 auto-regulatory feedback loops.

Our further investigation regarding involvement of DNA hypermethylation in dysregulation of p53-associated proteins, on the basis of demethylation therapy in breast cancer

cell lines (BT549, HS578T and MCF7) and *in-silico* pathway analysis, led us to characterize over-expression of Atic, Calr and Pcna proteins with regulatory role on p53 in wt-p53 breast cancer cell line (MCF7). These proteins regulate p53 function by different mechanisms; Atic has positive regulatory effect on p53 expression and phosphorylate p53 that leads to regulate cell growth and induction of apoptosis [9, 10]. Calr function is required for regulation and nuclear localization of the p53 protein and it also plays a role in inhibition of cancer metastasis [11, 12]. Pcna contributes to p53 stabilization via posttranslational modification[13, 14]. The low expression of Atic, Calr and Pcna in un-treated MCF7 might be explained by DNA hypermethylation, which was contributed to transcriptional silencing and eventually to down-regulation of protein expression.

The comparison of miRNA expression profiling of the wt-p53 breast cancer cell line (MCF-7) and mutant p53 breast cancer cell lines (BT549, HS578T) along with their interactions to the p53 signaling pathway, revealed differential expression for miR-125b, miR-15a, miR155, miR-16, miR-183 and miR-21, which could be suggested as p53 pathway disruptors in breast cancer. The previous studies in solid tumors defined different roles for these six dysregulated miRNAs in cancer such as in neoplasm progression (miR-125b, miR-15a and miR-16), invasion and metastasis (miR-155, miR-183 and miR-21) and they are also suggested to have prognostic values in cancer[15-19]. Our investigation on miRNAs disruptor in clinical specimens revealed significant dysregulation of miR-125b and miR-21 in the tumor tissues compared to the normal matched tissues ($P<0.05$) with *TP53* status-dependent manner for miR-21 ($P<0.05$). The down-regulation of miR-125b was inline with previous reports in breast cancer [20-22]. The pathway analysis presented regulatory connection between miR-21 and *TP53,* however, the binding site on the p53 mRNA for miR-21 has not yet been characterized and regulation of p53 by miR-21 or vice versa is a controversial issue [23, 24] and needed to be investigated in more depth.

To determine the mechanistic link between inactivated p53 related pathways through epigenetic silencing and the actual down-stream alterations in cancer kinetics, we assessed telomere length shortening and mtDNA damages, including D-loop region integrity and mtDNA content, in breast cancer patients. The telomere length and promoter methylation status of the *P16*/*Rb* and *TP53*/*P21* pathways were examined in breast cancer patients. Our results showed

that the average telomere length in breast cancer tissue was significantly shorter than that in the adjacent normal tissue, (P<0.001). Indeed, hypermethylation of *TP53*, *P21* and *P16* promoters significantly correlated with shortened telomere length in the cancer tissues (P<0.001). In general, malignant tumors show shorter telomeres than corresponding normal tissue, and telomere dysfunction has been indicated as a negative prognostic marker in solid tumors, including breast cancer [25, 26]. Telomere shortening in primary human cells leads to replicative senescence, which is regulated in part by effectors in the *P16*/*Rb* and/or *TP53*/*P21* pathways [27-29]. As well, p53/p21-mediated DNA damage signals are elicited by telomere dysfunction. However, there is still considerable debate on the exact role of telomere attrition on the *P16* pathway during senescence. Taken together, these findings suggest that *P16* and *P53*/*P21* may act as a gatekeeper to prevent critical telomere shortening and genome instability.

A part from aberrant DNA methylation and telomere length shortening, we found considerable mtDNA damage in tumor tissues, which was associated with the hypermethylated p53 auto-regulatory feedback loops (*P14* ^{ARF} and *PTEN*). Our data presented a high rate of D-loop region mutations (36.36% somatic and 90.91% germline mutations) in breast cancer patients. Association of *TP53* alteration with mtDNA damage has been reported in a breast cancer patient [30]. Moreover, significant mtDNA depletion in the tumor tissues compared to the normal tissues has been found in our study ($P< 0.01$). It has been demonstrated that the depletion of mtDNA resulted from a loss of p53 function [31]. The mtDNA damage in the studied cohort, D-loop mutations and mtDNA depletion, might be explained by the indirect effect of p53 inactivation, *P14*^{ARF} and *PTEN* hypermethylation, on mtDNA vulnerability.

Aberrant methylation profiles and silencing of only a small subset of tumor suppressors and cancer related genes in both the primary tumor and lymph node metastasis has been investigated for breast cancer [32-35]. We implemented the methylation signature of the 12 breast cancer candidate genes (*APC, BIN1, BMP6, BRCA1, CST6, ESR-b, GSTP1, P14, P16, P21, PTEN* and *TIMP3*) by comparing lymph nodes metastasis to their matched primary tumor tissues and normal tissues from the same breast cancer patients. The quantitative methylation analysis of the 12 studied genes in the present cohort showed higher methylation proportion for the primary tumor tissue versus matched normal tissue and the differences were significant for *APC, BIN1,*

BMP6, BRCA1, CST6, ESR-b, P16, PTEN and *TIMP3* promoter regions ($P<0.05$, $P<0.05$, $P<0.01$, $P<0.0001$, $P<0.01$, $P<0.01$, $P<0.05$, $P<0.05$, $P<0.01$; respectively). Among the analyzed genes, *APC, BMP6, BRCA1* and *P16* showed higher methylation proportions in matched lymph node metastasis than the normal tissues ($P<0.05$ and $P<0.01$, $P<0.0001$, $P<0.05$; respectively). Present findings provided evidence of differences in methylation status between primary tumors and their corresponding matched lymph nodes and demonstrated methylation heterogeneity between primary tumors and metastatic lesion which are in line with previous reports described about primary tumor and metastasis in breast, gastric and colorectal cancers[36-38]. The pathway analysis revealed that *BMP6*, *BRCA1* and *P16* have a role in prevention of neoplasm metastasis (Fig.3). The relation of DNA methylation for *BRCA1* and *P16* with tumor recurrence has been reported with a high value in breast cancer patients[39]. Moreover, association of *P16* hypermethylation with cancer progression and lymph node invasion has been shown in different studies[40, 41]. The aberrant methylation signatures of these genes found in the metastatic lymph node could be serving as markers for screening metastasis in breast cancer.

In conclusion, the results reported in this study increase the understanding of p53 inactivation and impaired p53 pathway in wt-p53 breast cancer and our finding might provide a further clue to establish meaningful biomarkers, which could improve prognosis and therapeutic management of the breast cancer patients. Thus, we suggest aberrant methylation alterations of APC*, BIN1, BMP6, BRCA1, CST6, ESR-b, GSTP1, P14^{ARF}, P16, P21, PTEN* and *TIMP3* as well as overexpression of miR-21, mtDNA damage and shortened telomere length as tumor specific biomarkers in breast cancer. The contribution of aberrant methylation alterations of *BMP6, BRCA1* and *P16* genes in lymph node metastasis suggests implementing these genes as markers for screening metastasis for breast cancer patients. Such biomarkers will be needed to be additionally explored for the potential opposing functional effects of specific hypermethylated CpG sites on transcriptional activity as well as for absolute percent methylation cutoffs for each breast tumor type that would enable reliable utility of them in the clinical laboratory setting.

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